ESTCP
Cost and Performance Report

(CU-9713)

Explosives Detecting Immunosensors

September 2000

ENVIRONMENTAL SECURITY
TECHNOLOGY CERTIFICATION PROGRAM

U.S. Department of Defense

Report Documentation Page	*Form Approved* *OMB No. 0704-0188*

1. REPORT DATE **SEP 2000**	2. REPORT TYPE	3. DATES COVERED **00-00-2000 to 00-00-2000**
4. TITLE AND SUBTITLE **Explosives Detecting Immunosensors**		5a. CONTRACT NUMBER
		5b. GRANT NUMBER
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)		5d. PROJECT NUMBER
		5e. TASK NUMBER
		5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U.S. Department of Defense,Environmental Security Technology Certification Program (ESTCP),901 North Stuart Street Suite 303,Arlington,VA,22203**		8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)		10. SPONSOR/MONITOR'S ACRONYM(S)
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)
12. DISTRIBUTION/AVAILABILITY STATEMENT **Approved for public release; distribution unlimited**		
13. SUPPLEMENTARY NOTES		
14. ABSTRACT		
15. SUBJECT TERMS		

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**74**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

Page

LIST OF FIGURES

Page

LIST OF TABLES

LIST OF ACRONYMS

ABG	Ammunition Burning Ground at NSWC
Analyte 2000	Fiber optic instrument by Research International, Inc.
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act of 1980 (a.k.a. Superfund)
CFI	Continuous Flow Immunosensor
DARPA	Defense Advanced Research Program Agency
DNT	Dinitrotoluene
EDA	Ethylenediamine
EPA	U.S. Environmental Protection Agency
ESTCP	Environmental Security and Technology Certification Program
FAST 2000	Continuous flow instrument by Research International, Inc.
FOB	Fiber optic biosensor
GAC	Granulated activated carbon
HMX	1,3,5,7-Tetranitro-1,3,5,7-tetrazacyclooctane
HPLC	High pressure liquid chromatography
MDL	Method Detection Limit
Method 8330	HPLC method for detecting explosives
NRL	Naval Research Laboratory
NSWC	Naval Surface Warfare Center
NSWC a.k.a. NAD	U.S. Naval Surface Weapons Center - Crane, Indiana Crane, NAVSURFWARCEN
ONR	Office of Naval Research
PAU	Peak area unit
PCMCIA	Personal Computer Multiple Computer Interface Accessory
QA/QC	Quality assurance, quality control
RCRA	Resource Conservation and Recovery Act
RDH	RDX hapten
RDX	1,3,5-Trinitro-1,3,5-triazacyclohexane
RI	Research International
RPD	Relative percent difference

LIST OF ACRONYMS (continued)

RP-HPLC	Reverse phase high pressure liquid chromatography
RQL	Reliable Quantitation Level
SD	Standard deviation
SERDP	Strategic Environmental Research and Development Program
SPE	Solid phase extraction
SUBASE	U.S. Navy Submarine Base - Bangor, Washington
TCE	Trichloroethylene
TNB	1,3,5-Trinitrobenzene
TNT	2,4,6-Trinitrotoluene
UMDA	U.S. Army Ammunition Depot - Umatilla, Oregon

ACKNOWLEDGMENTS

The authors would like to thank the following people and organizations who were involved with this demonstration project and the generation of this report.

- Naval Research Laboratory: Paul Charles; Charles Patterson, Jr.; David Holt; and Jeff Lundgren
- Geo-Center's Inc.: Paul Gauger, Irina Bakaltcheva
- George Mason University: Saskia van Bergen
- U.S. EPA Region 10: Harry Craig, Roy Araki
- Black and Veatch Waste Science: Ginger Ferguson, Andy Markos
- Naval Surface Weapons Center - Crane, IN: Tom Brent, Chris Freeman
- U.S. EPA Region 5: Carol Witt-Smith
- U.S. EPA Office of Solid Waste: Barry Lesnik
- U.S. Army Corps of Engineers, Waterways Experimental Station: Judith Pennington
- U.S. Army Corps of Engineers, CRREL: Tom Jenkins

This work was funded by Office of the Undersecretary of Defense Environmental Security Technology Certification Program (ESTCP). The views expressed here are those of the authors and do not represent those of the U.S. Navy, Department of Defense, or the U.S. Government.

Technical material contained in this report has been approved for public release.

This page left blank intentionally.

1.0 EXECUTIVE SUMMARY

To meet environmental remediation goals, there is a need for rapid, quantitative detection of hazardous pollutants such as explosives. Biosensors provide a rapid, specific, sensitive, portable, and inexpensive means to fulfill those needs. The Naval Research Laboratory has developed two methods for measuring TNT and RDX. These methods employ either the Analyte 2000 or the FAST 2000 optical instruments, both engineered by Research International (Woodinville, WA) in collaboration with NRL (Figures 1 and 2). These biosensors, based on fluorescence immunoassay techniques, are interfaced to portable computers for instrument control and data analysis. Both biosensors are portable, and easily set-up within 30 minutes on a small table. The Analyte 2000 is a fiber optic biosensor capable of simultaneously monitoring four optical probes. It is based on a competitive fluoroimmunoassay, in which a fluorescent molecule, similar to the analyte, competes with the analyte for binding sites on antibodies immobilized on the surface of an optical probe. In this format, the fluorescence signal is inversely proportional to the amount of analyte in the sample. Results are determined in 12-17 minutes depending on the analyte. Multiple analyses are performed on the same fiber probe to reducing probe to probe variation issues for quantitation.

Figure 1. Analyte 2000.

Figure 2. FAST 2000.

The Fast 2000 is a continuous flow immunosensor based on a displacement immunoassay, with the key components being antibodies specific for the analyte immobilized on a membrane support, fluorescent signal molecules similar to the analyte saturated on the immobilized antibodies, and a fluorescent detector. Upon injection of an explosive contaminated sample, fluorescent signal molecules are released into the flow stream and detected by a detector. The FAST 2000 quantitates samples with minimal sample preparation and reagent addition. Analysis is complete within five minutes, with the fluorescent signal being proportional to the analyte concentration in the sample.

To demonstrate these methods, extensive field trials (three for groundwater and one for soil), were conducted at several geochemically diverse sites. The groundwater sites, SUBASE Bangor (Washington), Umatilla Army Depot (Oregon) and NSWC Crane (Indiana), are on the U.S. EPA Superfund list. Additional soil samples from several sites were supplied by T. Jenkins (Cold Regions Research and Engineering Laboratory). Data was used to test detection limits (5-10 ppb in groundwater and 50-100 mg/kg for soil), reproducibility, bias, precision, calibration, waste generation, and matrix effect on detection limits. A cost analysis for the methods was also done.

Comprehensive laboratory tests were performed to determine cross-reactivity and false positive/ negative rates. In addition to the validation studies, limitations and appropriate scenarios for application of the methods were evaluated.

Overall, results for the biosensors suggest that the instruments are promising field technologies that will require additional development before they are suitable for field use. The instruments were simple to use, required minimal sample preparation, were easily carried to the field and generated minimal waste. Determinations of TNT and RDX levels in spiked water samples were accurate and precise down to 10 µg/L, with acceptable levels of false positive/false negative values. However, significant problems were encountered with respect to accuracy and precision in environmental sample measurements. In general, the biosensors were predictive and gave similar yes/no results as the direct injection protocol of U.S. EPA SW846 Method 8330 (high performance liquid chromatography) at the field detection limit of 20 µg/L . Site-specific matrix effects produced a large scatter in data points, with a lower level of agreement to HPLC quantitative values for several data sets when compared to the field spike results. Of particular concern was the large number of false positive values for the TNT assay. Further development of the technologies will focus on improved assay performance in environmental matrices, sample preparation for low-end detection, and improved signal processing and instrument calculations to remove user bias.

2.0 TECHNOLOGY DESCRIPTION

2.1 FIBER OPTIC BIOSENSOR

The fiber optic biosensor (FOB) uses molecular recognition and evanescent wave sensing to detect a wide variety of analytes. (Ref. 1-9) The fiber optic sensor consists of a multichannel “fluorimeter”, a fiber bundle jumper, and disposable fiber optic probes. (Ref. 10) Properties of optical fibers provide a mechanism for exciting fluorescent molecules that are very close to the fiber core. Light is totally internally reflected within the optical fiber core and an electromagnetic field is generated around the core with power that decreases exponentially with distance from the core surface. This field is referred to as the evanescent wave (Figure 3). The effective or penetration depth of this field is determined by the wavelength of light and the refractive indices of the fiber core and the surrounding media. In the case of the FOB, the penetration depth is approximately 125 nm. Fluorescent molecules that enter the evanescent wave are excited and emit light at a longer wavelength, i.e., fluorescence. Effectively, these fluorescent molecules are ones that bind to the surface, i.e., antibody-fluorescent analyte complexes. A portion of this fluorescence is captured by the fiber and transmitted to a detector. Molecules outside the evanescent wave are not detected by the sensor, thereby eliminating wash steps.

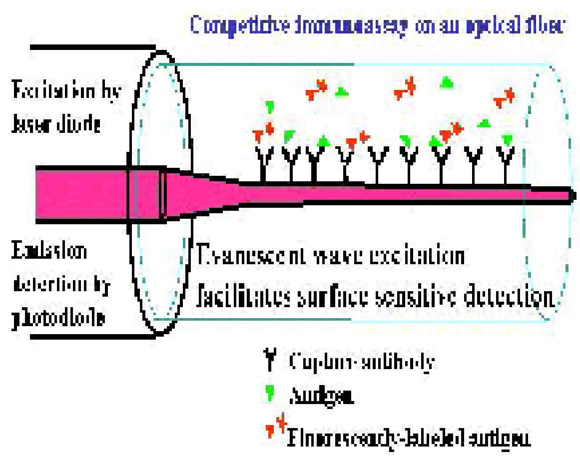

Figure 3. Schematic of the Optical Fiber.

The multichannel “fluorimeter” Analyte 2000, produced by Research International in collaboration with NRL (Figure 1), consists of four integrated circuit ‘daughter’ cards that are monitored by a microprocessor-based controller board. (Ref. 10) On each ‘daughter’ card is mounted a 5 mW 635 nm diode laser modulated at 135 Hz for synchronous detection. An internal transfer fiber transmits the laser light to the excitation leg of the bundle jumper. A second internal fiber transmits the fluorescent emission from the bundle jumper to a photodiode. Appropriate filters and signal calibration controls are also incorporated on each ‘daughter’ card. The controller board monitors each card and sends the measured signal from each channel to a laptop computer through an RS-232 communication port. The computer software collects, plots, stores data, and permits user control over several other functions. The fiber bundle jumper transmits the excitation light from the “fluorimeter” to the fiber optic probe and the returning fluorescent signal to the device. The fiber optic probe provides the sensing region for the biosensor. Each optical probe is made from 600 µm diameter fused silica multimode

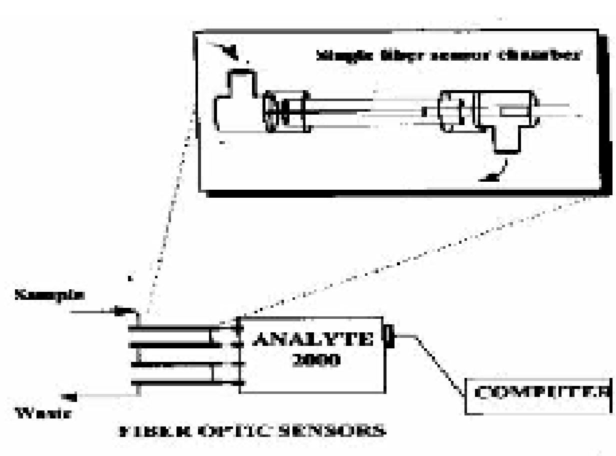

Figure 4. Layout Design of the Analyte 2000.

fibers. One end of the probe has the cladding removed to permit attachment of the recognition molecule directly onto the fiber core. This sensing region is tapered to provide efficient fluorescence excitation and signal collection. (Ref. 11) After the recognition molecule is immobilized, the coated probe is inserted into a sample chamber formed from a 100 µl capillary tube with plastic t-connectors on each end (Figure 4). (Ref. 8) The capillary chamber system can be injected with syringes or peristaltic pumps for system automation. A semi-automated fluidics system developed at NRL, which employs a mini peristaltic pump, was used for this study.

The disposable fiber optic probes provide the region for specific detection with antibodies, immobilized on the surface of an optical fiber providing the molecular recognition. Degree of specificity is determined by the choice of the antibody employed. For small molecules such as TNT and RDX, a competitive fluoroimmunoassay is performed. In this assay, a fluorescently-labeled analyte analog competes with the analyte for antibody binding sites. A decrease in the maximum fluorescent signal is observed that is proportional to the analyte concentration.

The fiber optic biosensor system is rapid (<17 min including reference), reliable, portable, and highly sensitive (ppb), and can be used to detect substances in real-world samples such as river water, groundwater, leachate and bilge water and in soil extracts. We have demonstrated successful analyses in opaque, viscous samples with a portable fiber optic sensor. This portable sensor is also capable of detecting four test samples simultaneously.

2.2 CONTINUOUS FLOW IMMUNOSENSOR

The Continuous Flow Immunosensor (CFI) is based on a displacement assay that utilizes antibodies as a means of detection. The key elements of the sensor are: (1) antibodies specific for the analyte, (2) signal molecules which are similar to the analyte but labeled with a fluorophore (usually a Cy5 dye) so they are highly visible to a fluorescence detector, and (3) a fluorescence detector. For an analysis, the antibodies which specifically recognize the contaminants are immobilized onto a solid support and saturated with the fluorescently labeled signal molecule, creating an antibody/signal molecule complex. The functionalized support is placed in the sensor and connected to a buffer stream. For the FAST 2000, a sample is introduced to the system through the injection port. If the sample contains the target analyte, a proportional amount of the labeled signal molecule is displaced from the antibody and detected by the fluorimeter downstream. Figure 5 shows a schematic of the immunosensor operation. Displacement assays, using the laboratory version of the CFI, have been developed for a wide range of small molecular weight compounds, including drugs, explosives, and pesticides. (Refs. 12-17)

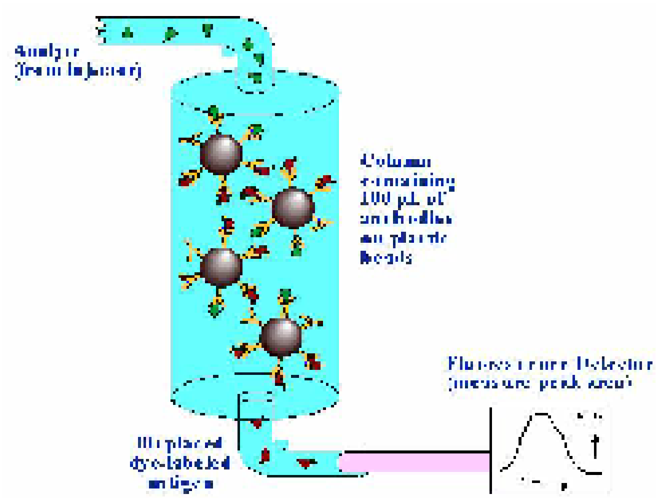

Figure 5. The Continuous Flow Immunosensor (CFI).

The manufacturable, field-portable version of the CFI, the FAST 2000, has been engineered by Research International (Figure 2). The

FAST 2000 requires a computer capable of running Windows 95 or Windows 3.1 in enhanced mode. The FAST 2000 is a rapid and convenient system for performing displacement assays with low ppb explosive levels in water and soil. The optically-based signal gathering capabilities are combined with precise fluidics control in a PCMCIA-based PC application. The unit can be easily carried into the field and plugged directly into a portable PC for on-site data acquisition and analysis. Analysis time for each sample is approximately 2 minutes. The system is controlled by an advanced Windows-based software program while the hardware is designed to use a National Instruments data acquisition card (DAQ Card - 1200) for gathering data. An outboard box, connected to the FAST 2000 unit via color-coded tubing, contains the waste bottle and buffer bag.

The system has been developed as a complete turnkey unit using advanced Windows-based software program to control the system. The software provides a simple menu driven interactive interface to lead users through the steps required to successfully determine if a trace amount of analyte is present in a given sample. The software also allows the more advanced user complete control of the operational parameters for running nonstandard procedures. The hardware provides the necessary fluid storage and flow control.

The FAST 2000 system utilizes a disposable coupon for performing the assays. The coupon contains discrete flow channels, a membrane and filter pocket in a removable plug, pneumatically controlled valves, and septum seal area used for injecting fluids into the coupon. The coupons are assembled with the functionalized membranes before shipping. Prior to instrument operation, the coupon is inserted into the FAST 2000 control unit, and when the handle is engaged, the coupon septum is automatically pierced. Through the Task Manager in the system software, assays are performed by a sequence of valve controls which meter the assay fluids through the coupon and into the membrane pocket. The user is instructed when to inject the sample into the small septum area on the top of the coupon with the needles of a small volume syringe. The sample volume required to perform a single assay is 0.15 mL. The fluids then exit the coupon and travel into the integral fluorimeter in the control unit which detects any fluorescence signal present.

Data analysis is made easy with the use of real time plotting of the data, data logging, and custom calibrations. The Windows-based software allows for both ease of use and complex system manipulation, keeping all skill levels in mind. The assay chemistry for TNT and RDX detection has been developed to be a system that can be successfully used in the field without the need for excessive environmental controls. Quantitation of the analytes, done by the system software, compares fluorescence intensity to that of a standard.

The coupon and membrane can be used for repeated assays. The life of the membrane is dependent upon the number and concentration of positive assays that were run. Since only a limited quantity of the label is bound to the antibodies on the membrane, it will eventually become depleted of the label. Membranes that need to be replaced will have significantly reduced signal peaks and the baseline will be less than 400 pA on a scale of 0-2400pA. This may take one to three days. If a standard sample cannot be detected, the membrane must be replaced.

2.3 FIBER OPTIC BIOSENSOR AND CONTINUOUS FLOW IMMUNOSENSOR COMPARISON

The FOB and the CFI are both technologies that rely on antibody-analyte interaction, with fluorescence used for signal transduction. However, they are complementary rather than competing methods, with applications in distinctly different areas. Table 1 summarizes the differences and similarities discussed in previous sections. Specifically, the FOB is more suitable for testing environments requiring remote detection (i.e., soil or groundwater monitoring with a cone penetrometer). In contrast, the CFI is more appropriate and cost effective in test scenarios that require routine on-site measurements of either discrete samples or intermittent monitoring of process streams (pump-and-treat filters, quarterly tests of monitoring wells). In either case, both sensors are rapid compared to current technologies and are easy to set up and operate in the field. The choice of which sensor to employ must be decided by remediation managers on a case-by-case basis.

Table 1. Fiber Optic Biosensor/Continuous Flow Immunosenor Comparison.

FOB	CFI
Competition Immunoassay 4 simultaneous assays 8-16 min/assay Cone penetrometer monitoring	Displacement Immunoassay Sequential assays 2 min/assay Intermittent on-line monitoring
Rapid Simple set-up Field portable Field tested TNT and RDX assay	

2.4 STRENGTHS, ADVANTAGES, AND WEAKNESSES

The FOB and the CFI are rapid analytical tools for the on-site detection and monitoring of compounds. Little sample volume or manipulation is required for detection with the exception of an acetone extraction for soil samples. The biosensors are completely portable (battery operated and lightweight), which is preferable for on-site analysis. Full set-up (from shipping box to sample analysis) takes approximately 1 hour.

The major strength of the NRL sensors is their adaptability for use in a variety of environments. The biosensors have been tested directly in a variety of environmental media including ground and river water, leachate, and soil extracts, that may or may not contain particulates with some site specific effect, on the overall activity of the sensors. Samples can be injected by hand or pump from air samplers that extract vapors into water, or soil extractions. In addition, super sipper systems that rapidly inject samples from hundreds of vials can be employed.

The FOB is capable of analyzing a single sample run either in quadruplicate over four similar fibers or four fibers with different antibodies simultaneously. This advantage provides the ability to have assay controls performed during sample analysis. In the case of TNT, the fiber probes have been "regenerated" and reused up to 16 test samples. The CFI can be used either for continuous monitoring of a water stream, or for testing multiple discrete samples sequentially for an extended

period of time per antibody cartridge. The number of samples tested is based in part on the number and concentration of positives, since negative samples do not deplete the labeled analyte from the cartridge. For TNT and RDX, more than 50 positives can be analyzed over a single column/ membrane.

The detection limit of the instruments for laboratory samples is already comparable to established, more complicated systems. Using the NRL sensors, TNT and RDX in water has been detected at levels of less than 5 parts per billion in buffer (equivalent to 5 ng/mL) in the laboratory. This level of sensitivity is well-below that obtained using precipitation, dip stick, most enzyme immunoassays, and fluorescence polarization methods, and is comparable to radioimmunoassays. However, from these studies, it was determined that the limit of detection for field samples is slightly higher (5-20 ppb in groundwater and 50-100 ppb for soil) than the laboratory spikes. This decrease in sensitivity and associated matrix effect may, at times, compromise assay performance.

Antibodies are recognized by biochemists and molecular biologists for their exquisite specificities. Antibody selection is based on affinity and specificity for the compound of interest. Antibodies can be selected such that the specificity is a narrow range for just one compound or wider for a group of similar compounds. Closely related compounds may also react with the antibody but usually with a lower affinity. Molecules such as TNT and RDX are too small to be antigenic so they or a closely related analog is coupled to a larger protein for antibody production. A larger protein cannot be coupled directly to TNT so the compound trinitrobenzene (TNB) was linked to a protein and used as the antigen to elicit antibody production. The TNT antibody used with the fiber optic biosensor, obtained from Strategic Diagnostics, Inc. (Newark, DE), was produced against a TNB conjugate and selected for its affinity for TNT. Therefore, this antibody reacts with both TNT and TNB. The same is true for the 11B3 anti-TNT antibody employed in the continuous flow immunosensor. (Ref. 12) This cross-reactivity poses a problem if one needs know the exact concentration of TNT in the presence of TNB. The result would be an overestimation of TNT in the sample. However, since both TNT and its degradation product TNB are both toxic and explosive, this cross-reactivity is not necessarily a detriment with a screening system as both require cleanup/remediation. The RDX antibody used with both sensors, obtained from Strategic Diagnostics, Inc., has also been selected for its strong affinity and low cross-reactivity with other compounds. The extent of its cross-reactivities is detailed in the company brochure but does include HMX.

One problem with any antibody-based assay is that the compound of interest must be known prior to analysis so that the appropriate antibody can be employed. Unlike HPLC which identifies a large number of compounds, an antibody recognizes only single or limited numbers of compounds. Most samples contain both toxic and nontoxic components. In HPLC, both types will be identified with possible swamping of the toxic compounds by the nontoxic ones unless a laborious extraction procedure is followed. This problem can be eliminated using antibody-based assays because only the toxic compound generates an antibody-mediated signal.

The antibody-antigen reaction is not a covalent one but one of structural complementarity. The binding is comprised of hydrophobic and electrostatic interactions. Since these are not permanent bonds, conditions in real world samples can disrupt those interactions. Examples of such conditions include the presence of cross-reactant compounds, extremes in ionic strength of sample, pH of sample, humic materials, and competitors for the antigen. If it is determined that real world matrix

interferes with the antibody-antigen reaction, there are several solutions available including filtering, solid phase extraction and solution buffering.

Antibodies have proven to be very reliable, sensitive and specific for detection for clinical applications. The clinical matrices are quite complex as are the environmental matrices for which these sensors are proposed to utilized. The strengths of antibodies seem to outweigh the weaknesses.

The following factors need to be considered in evaluating the cost and performance of the immunosensors: commercial production, training, and availability. The technologies are just now coming on the market and have not been widely tested. The Analyte 2000 fiber optic biosensor is being marketed by Research International for less than $20K, but the fiber probes are not yet in commercial production. Currently, a technically trained person is required to make the fiber probes and operate the system. However, prototypes of an automated version of the fiber optic biosensor have been fabricated and are currently being tested. The Fast 2000 continuous flow sensor, while similar in cost to the fiber optic sensor, is available from Research International without the antibody-coated membranes. The coated membranes are not commercially available at this time but RI is pursuing this issue. Background experiments and previous laboratory studies were done using a noncommercial version of the system built at NRL.

3.0 DEMONSTRATION DESIGN

3.1 PERFORMANCE OBJECTIVES

The objectives of the field trials were the demonstration of the biosensors being operated on-site by non-NRL personnel as well as NRL staff and the generation of analytical data appropriate for sensor validation and certification by a regulatory agency such as the U.S. EPA or Cal EPA. A minimum of four instruments of each biosensor type was employed for each field trial.

A specific goal for the NRL environmental immunosensors was to achieve 1-5 ppb sensitivity for TNT and RDX in environmental groundwater samples and 50-100 ppb in soil samples. Specificity of the sensors was provided by the antibodies immobilized on solid matrices within the biosensors. The immunosensors should be specific for TNT and RDX with minimum cross-reactivities. It should be noted that cross-reactivity with TNB and HMX are expected with the antibodies employed. Accuracy and precision were evaluated using linear regression and relative percent differences (RPD). It has been noted in several papers that ± 50% RPD is routinely used as the control limit. (Refs. 18,19) Our goal for the linear regressions was a slope significantly different from zero with 95% confidence (assessed by t-test). A student's two-tailed paired t-test (a test of accuracy) and the Fisher F-test (a test of variance) at the 95% confidence level were performed on all field trial data values. In each case, the goal was to obtain values that indicate no significant difference between the immunosensors and Method 8330 could be demonstrated. The field data was also evaluated for false positive/false negative rates with the goal of having <10% false positive and 0% false negative. In addition to sensitivity and specificity, other advantages of the sensors including low generation of waste, short analysis times, limited sample preparation, low cost per analysis, and little or no matrix effects were validated.

To meet these objectives, three field trials for groundwater analysis and one for soil were performed using the two biosensors to perform on-site analysis. The first groundwater test for this project was conducted in June 23-27, 1997 at SUBASE Bangor, Bangor, WA. The second site was Umatilla Army Depot (UMDA) in Hermiston, OR from August 4-8, 1997. The third site was Naval Surface Weapons Center in Crane, IN from September 8-12, 1997. The soil field trial was held April 27-May 1, 1998 at Manchester, WA on samples from Umatilla Army Depot. Both sensors were operated on-site by non-NRL employees as well as NRL staff. Splits of the field sample were analyzed by the immunosensors and U.S. EPA SW846 Method 8330. In addition to on-site soil analysis, Tom Jenkins of CRREL provided ten archived soil samples from various sites in the U.S. The biosensor results for the field samples were evaluated on accuracy, precision, false positives/negatives rates, predictability, cost, time, and waste generation. Samples from other contaminated sites were also analyzed to study groundwater matrix effects. In addition to the contaminated field samples, appropriate controls, blanks, laboratory spikes and cross-reactants were tested in the laboratory for certification and validation data requirements.

3.2 PHYSICAL SETUP AND OPERATION

The physical setup was similar at each site. The biosensors were setup in a room (usually a conference room) at the test site. The buildings were temperature controlled and electricity was available. A refrigerator and sink were located near the room employed for testing. Samples were kept cool in coolers if refrigeration was not in the test room. All preparation of the samples was

performed in the room with the instrumentation. All materials necessary for the analysis of groundwater were carried with us on-site. Setup of the four flow immunosensors took approximately 30 minutes. The antibody-coated membranes need to washed prior to initial sample analysis to obtain a sample baseline. The four fiber optic biosensors were operational in less than one hour. Deionized water (purchased from the local grocery store or the U.S. EPA laboratory at SUBASE Bangor) was used at all sites.

3.3 SAMPLING PROCEDURES

3.3.1 Groundwater

Groundwater from monitoring and extraction wells in contaminated areas were collected by on-site personnel or EPA Region 10's contractor for analysis. In addition, spring water was also collected at the Crane site. Samples were initially collected into 20L EPA-approved cleaned containers and sealed until on-site analysis or shipment to laboratories for analysis. In addition, groundwater samples were collected from the combined flow from the extraction wells at sampling ports before and after initial particulate filters and upstream of the granular activated carbon (GAC) unit at SUBASE Bangor. Aliquots or splits from the large sample container were used for laboratory and field analysis. These aliquots (one liter for each laboratory and 40 mLs for on-site analysis by the biosensors) were stored in EPA-approved cleaned amber bottles in the dark and cool (4°C). Due to rapid TNT degradation in groundwater, analysis for TNT was performed within one month of collection. The contract laboratories were monitored by Harry Craig of U.S. EPA Region 10 (QST, Gainesville, FL) and P. Gauger of Geo-Centers, Inc. (GP Laboratories).

3.3.2 Soil

Soils from Umatilla Army Depot were provided by H. Craig (U.S. EPA) and Gannett Fleming staff. Additional soil samples were provided by T. Jenkins (CRREL). The locations of the additional soil samples were Ft. Ord, CA (1), Hawthorne Army Ammunition Plant, Hawthorne, NV (3), Raritan Arsenal, NJ (1), and Nebraska Ordnance Plant, Mead, NE (4). They were archived samples that were dry, well homogenized, and fully characterized.

3.4 ANALYTICAL PROCEDURES

3.4.1 Soil Extraction

An acetone extraction was performed on all soil samples. (Ref. 19) For the on-site field trial, 20 gm of soil was mixed with 100 mL acetone. The sample was shaken for three minutes and then filtered. The acetone extract was measured. The extract was stored in amber containers at 4°C until analysis. Since there was less than 5 gms of the archived soils, the procedure was modified to 2 gm of soil and 10 mL acetone.

3.4.2 Fiber Optic Biosensor

Detection of TNT and RDX was achieved by performing competitive fluorescence immunoassays on the surface of an antibody-coated fiber probe. (Ref. 12) The procedure for making the antibody-coated optical probes has been described in detail. (Ref. 11) The antibody-coated fibers can be

stored for > 1 year before use. The preferred method for storage is lyophilized or in buffer at 4°C, but they can be stored for extended periods at 25°C.

In a competitive fluoroimmunoassay like the one for TNT and RDX, a fluorescent compound competes with the unlabeled compound in the sample for the limited number of antibody binding sites. The maximum fluorescent signal occurs when there is only the fluorescently-labeled compound present. Fluorescently-labeled TNB (Cy5-EDA-TNB) was used as the competitor in the TNT assay and fluorescently-labeled RDX hapten (Cy5-EDA-RDH) for RDX. (Ref. 9) As the unlabeled compound increases, a proportional decrease in the fluorescent signal is observed. Using a standard curve generated by evaluating known concentrations of unlabeled compound on the FOB, unknowns can be assayed and the results compared to the standard curve to determine the concentrations in the test sample.

In the TNT assays run during the field trials, all test solutions, reference solutions and controls contained buffer with the following components: 7.5 μg/L Cy5-EDA-TNB in 1x PBS pH 7.4, 5% acetone, 2 mg/mL bovine serum albumin and 0.1% Tween 20. A 10x stock solution of this buffer was used to make all test solutions. After a background reading from PBS buffer, a solution containing only the Cy5-EDA-TNB (reference solution) was exposed to an antibody-coated optical fiber probe for five minutes. Upon laser excitation of the fiber probe, a specific signal that corresponded to the maximum (100%) or reference signal was generated. This reference signal is defined as the signal change associated with the labeled TNB alone. The fiber probe was washed with 50% ethanol in buffer for five minute to remove the Cy5-EDA-TNB. In the case of explosives, the explosive and the labeled analog are more soluble in the ethanol solution than the buffer. This fact along with the moderate affinity of the antibody permit removal of the material bound to the fiber probe. Next, the probe was re-equilibrated with the PBS buffer solution for two minutes to prepare it for the next sample.

An unknown or standard is then assayed in a protocol identical to the reference solution. To the unknown or standard, fluorescently-labeled TNB is added to make the sample contain the same concentration as that used for the reference sample (7.5 μg/L Cy5-EDA-TNB). For water studies, the groundwater replaces deionized water in preparation of the sample. For soils, the acetone extract is employed to achieve the 5% acetone component of the sample, thereby creating a 1:20 dilution. Additional dilutions of the acetone extract may be required to obtain a reading that falls on the standard curve. The fluorescent signal for the test sample should be lower than the reference signal if TNT is present. After the test sample, the fiber probe was regenerated and re-equilibrated with PBS buffer. The protocol for analysis was a reference assay (Cy5-EDA-TNB only), regeneration of the fiber, test sample assay, regeneration, and then another reference assay. If multiple test samples were being assayed consecutively, only a single reference assay is run between test assays.

The RDX competitive immunoassays followed the same procedure with the following exceptions. First, Cy5-EDA-RDH is employed in place of Cy5-EDA-TNB but at the same concentration. The second exception is the length of time for regeneration. The fiber optic probe is exposed to the 50% ethanol solution for ten minutes instead of the five minutes. This is due to the relative affinity for the fluorescent conjugate of the anti-RDX antibody compared to the anti-TNT antibody.

Inhibition of the reference signal was observed when TNT or RDX was present in the test sample. The percent inhibition observed was proportional to the explosive concentration in the sample. The

reference signal value was determined both before and after the test sample assay in order to normalize for the gradual decrease in the antibody activity. Equation 1 was used to determine the percent inhibition of the 100% signal value by TNT or RDX.

$$\textbf{\%}\,\mathit{Inhibition} = \left[1 - \left[\mathit{Test\ signal} / \left(\left(\boldsymbol{Reference_{pre}} + \boldsymbol{Reference_{post}}\right) / 2\right)\right]\right] * 100 \quad \textbf{Eq. 1}$$

By employing the standard curve, the unknown samples could be converted from percent inhibition to µg/L (ppb). The % inhibition and concentration values were determined for each analysis and there was a minimum of seven fiber probes analyzes per test sample.

3.4.3 Continuous Flow Immunosensor

The CFI is based on a displacement immunoassay in which an explosive molecule in the sample selectively "displaces" a fluorescently labeled signal molecule from an immobilized antibody. This sensor has been described extensively in the literature based on work with the laboratory version. (Ref. 15) Procedures used in the field trials with the new FAST 2000 portable instrument were modified from previously published work to reflect differences from the laboratory sensor operation. In the new portable unit, all assay parameters and commands are controlled using a PCMCIA-based PC software program. The NRL's 11B3 TNT and Strategic Diagnostics' RDX monoclonal antibodies were immobilized onto porous membrane supports and saturated with the fluorescent analog. The membrane was inserted into a disposable coupon, the coupon was placed in the FAST 2000, and the buffer flow was started. Once the fluorescence background signal due to unbound CY5 had stabilized (generally 15-20 minutes), the biosensor was ready for sample injection. Samples of 150 µl were injected using a 1cc tuberculin syringe in the following order: standard (100-1000 ppb), three test samples, standard, two test samples, standard, two test samples and a standard. The last three standards should be in the range of the test sample. This injection protocol proved to be close to ideal when dealing with the displacement assay where fluorescence peak area decreases both with subsequent samples and time. By comparing standard injections at the beginning of the sample run with the middle and end of the run standards, we were able to monitor membrane behavior and change the membrane before the accuracy of the analyses was compromised. Also, standards could be selected that closely matched the concentration of the sample. This calibration method improves as working experience with the instrument increases, but even the non-developer users quickly understood how the instrument was behaving and could select standards that closely matched the samples.

For all samples, the computer calculated the Peak Area (PA) from points that corresponded to the beginning and end of the peak, as defined by the operator. To calculate a sample concentration, the peak area value for each sample was compared to the calibration standards injected before the sample. Ideally, the standards concentrations were close in value to signals obtained from the samples being analyzed. This value was then used to derive a concentration/unit signal value [ng/mL/Peak Area Unit (PAU)]. The averaged value was then applied to each PA from each sample injection to acquire a concentration for that injection of the sample. The concentrations were averaged and the Standard Deviation (SD) was calculated. In some cases, outlying values were rejected using the Q-Test with a 95% confidence rejection criterion.

3.4.4 SW-846 Method 8330

The EPA-approved method for explosive analysis in groundwater is SW-846 Method 8330. This method employs high performance liquid chromatography (HPLC) and a UV detector to determine explosive concentrations. For low concentration samples (< 20 µg/L), a salting-out extraction was performed, whereas higher concentration samples were injected directly. All analysis by QST and GP Laboratories on the test sample splits and standards employed the salting-out extraction step prior to analysis. In addition to the contract laboratories, NRL performed direct injection analyses of all samples. The columns for HPLC analysis were a C-18 reverse phase followed by a CN reverse phase column. The mobile phase was 50/50 (v/v) methanol/sample or methanol/water. The absorbance was monitored at 254 nm. The explosive concentrations for Method 8330 were based on a single analysis, unlike the multiple analyzes performed by both biosensors.

3.5 DEMONSTRATION SITE/FACILITY BACKGROUND

Site selection was based on several criteria including contamination with explosives, accessability to the site and the groundwater, U.S. EPA interest (i.e., Superfund), availability of non-NRL personnel and variety in geochemical parameters. Samples from three sites in the continental United States were analyzed on-site with the biosensors. Two of the facilities (SUBASE Bangor and Umatilla) are currently undergoing extensive remediation for groundwater contamination with TNT and RDX using pump-and-treat technology. As a result, these sites provided a number of platforms for effective testing of the sensors, including (a) direct measurement of contamination levels in monitoring wells, (b) analysis of samples in the treatment system (pre- and post-GAC filtration), (c) direct comparisons with current field and lab measurements using the ENSYS test kit and SW 846 Method 8330, respectively, and (d) experienced Army Corps of Engineers personnel familiar with the site. The EPA Region 10 military site coordinator (two of the sites are in Region 10) provided non-developer personnel to run tests, in compliance with the validation guidelines, as well as assisted in obtaining necessary logistical support.

3.5.1 Naval Submarine Base - Bangor, Washington

Naval Submarine Base (SUBASE) Bangor, located northwest of Seattle, Washington, is currently the home port for Trident submarines. From 1942 to 1973, SUBASE Bangor was used as an ammunition depot. Two sites (Site A and Site F) on the base have been inactivated due to explosive contamination. Wastewater from ordnance demilitarization was disposed into an unlined lagoon (Site F). Currently this site is undergoing cleanup via a pump-and-treat method through GAC filters. Sediment that accumulated at Site F was transported to Site A for burning and disposal in a lined area. Water is flushed through the contaminated soil, collected as leachate and processed through a different GAC unit. The four major contaminates identified are TNT, TNB, RDX, and HMX, ranging in concentration from 0-10,000 μg/L.

3.5.2 U.S. Army Ammunition Depot - Umatilla, Oregon

UMDA, located in eastern Oregon, is slated for closure. The base was established as an Army ordnance depot in 1941. From the 1950's until the mid-1960's, UMDA operated an explosive washout facility to remove and recover explosives from munitions. The standard and accepted procedure was to flush and drain the washout system into two unlined infiltration basins or lagoons.

A 45-acre plume of RDX in the shallow groundwater aquifer near the lagoons was identified in 1981. Further investigation documented the presence of explosives in both soil and groundwater, ranging in concentration from 0-10,000 μg/L in the groundwater aquifer. These explosives included TNT, TNB, RDX, and HMX. Bioremediation of the soils from the lagoons is currently underway. Treatment of the groundwater consists of pump-and-treat through GAC filters, with re-injection of the polished water back into the aquifer.

3.5.3 Naval Surface Weapons Center - Crane, Indiana

In late 1941, Burns City Naval Ammunition Depot (later renamed NSWC Crane) was established. The overall mission was to load, prepare, renovate, receive, store and issue ammunition to the fleet. Over the next few years, NSWC Crane's role increased to include pyrotechnics production, mine filling, rocket assembly, torpedo storage, ordnance spare parts, and mobile equipment storage. NSWC Crane supplied ammunition during the Korean and Vietnam conflicts to the fleet. In 1976, the mission was changed to provide support for ships equipment, shipboard weapon systems, and assigned ordnance items as well as provide support for the Crane Army Ammunition Activity which includes production and renovation of ammunition, storage, demilitarization and disposal of conventional ammunition. Contamination at Crane, located at three sites: (a) Ammunition Burning Ground (ABG) (b) Rockeye and (c) Rifle Range, is primarily due to the demilitarization and disposal of ammunition and pyrotechnics. High levels of trichloroethylene (TCE) are also present in these areas. Since the 1940's, ABG has been used extensively for destruction of explosive contaminated material. Between 1956 and 1960, 15,000 pounds/day of smokeless powder and 48,000 pounds/day of high explosives were burned. Initially, solid explosive residues were spread out on burning pads or in flash pits and ignited. Today, clay-lined steel pans are employed. For the liquid explosive contaminated material, three surface ponds were employed to remove the liquid from combustible sludge. In 1982, the ponds were modified to include a liner and leachate collection system. Currently, sludge burn pads are used and the ponds closed. Leachate and runoff were initially stored in two underground tanks. Now pink water is stored in two above ground tanks and the underground tanks are closed. Demilitarization continues with more stringent requirements to prevent soil and water contamination.

3.6 DEMONSTRATION SITE/FACILITY CHARACTERISTICS

3.6.1 Naval Submarine Base - Bangor, Washington (Groundwater)

SUBASE Bangor is located in a wet climate. The hydrology of the soils is fluvial/glacial deposition with high levels of organic compounds. The groundwater from the contaminated region is pumped to a facility containing several GAC units. Approximately 600 gallons of water per minute is treated with this system. The groundwater is known to be high in organic material and highly turbid. Figure 6 contains a map of SUBASE Bangor and the contaminated sites that were used for this field trial, Sites A and F, are highlighted. Site F was used for demilitarization and is the area where the unlined lagoons contained the wastewater. The groundwater from this area is undergoing remediation through GAC units. The water treatment facility is identified on this figure. Groundwater from the monitoring wells and pre-/post-GAC units was analyzed.

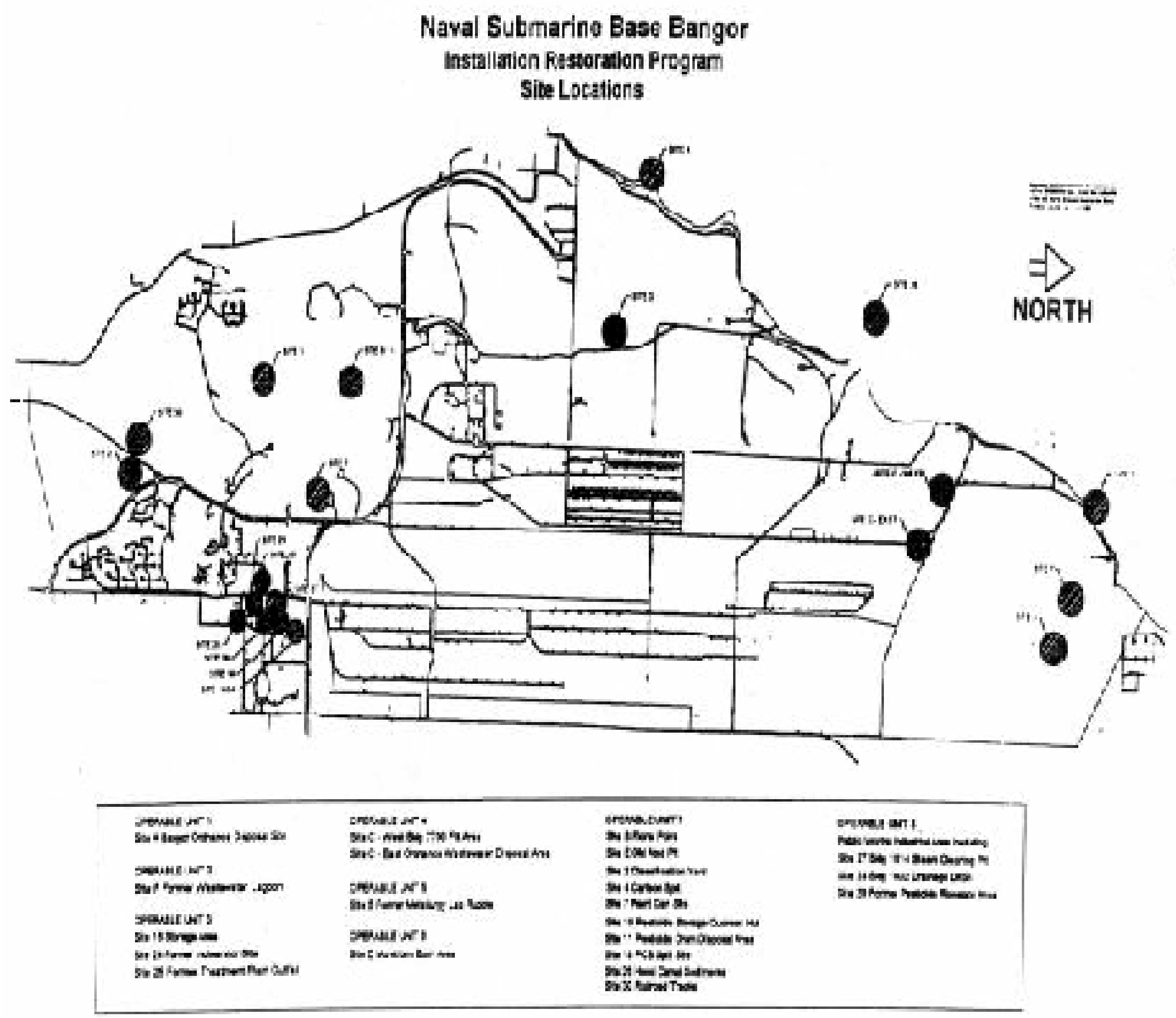

Figure 6. Map of Naval Submarine Base - Bangor, WA.

3.6.2 U.S. Army Ammunition Depot - Umatilla, Oregon (Groundwater and Soil)

UMDA is located near the Columbia River in an arid region with no surface water. The primary geology is alluvium on top of basalt, with approximately 100 feet to groundwater. The groundwater flow is northeast to southeast, depending upon the irrigation pumping season. The net flow to the southeast has led to the spread of explosive contamination. The groundwater from the contaminated region is pumped to a facility containing several GAC units. Approximately 600 gallons of water per minute is treated with this system. Figure 7 provides a map of UMDA and the contaminated sites that were used for this field trial. The site of the former munitions cleanout plant, now demolished, is marked “A”. The extent of contamination (approximately 45 acres) is shown by the concentric circles.

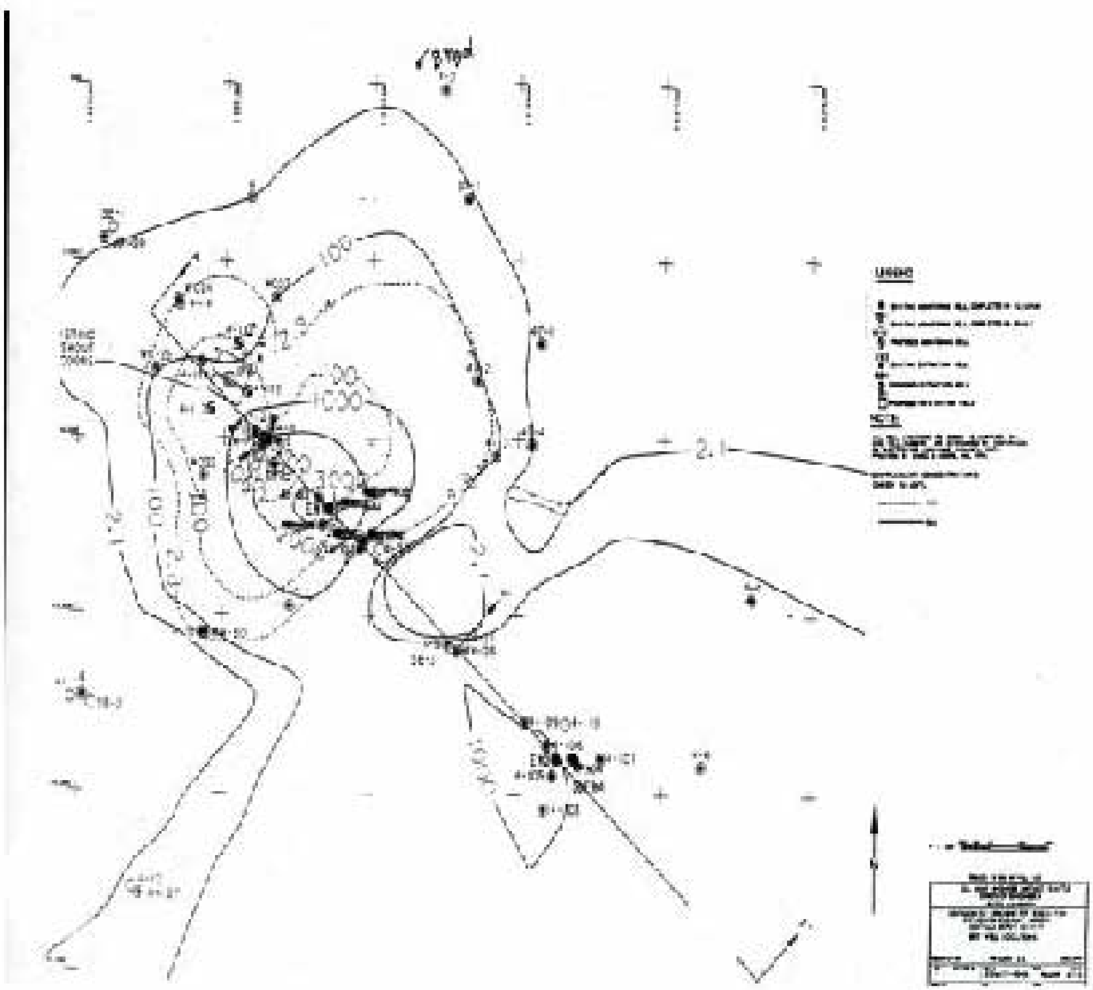

Figure 7. Map of U.S. Army Ammunition Depot - Umatilla, OR.

3.6.3 Naval Surface Weapons Center - Crane, Indiana (Groundwater)

NSWC Crane is located in the eastern Illinois Basin. Crane consists of undulating terrain with many small drainageways. Four types of soil are identified at Crane including Wellston-Gilpin, Wellston-Berks-Gilpin, Wellston-Berks-Ebal and Wakeland-Wilbur-Haymond. These soils are primarily silt loams. The bedrock at Crane is lower Pennsylvanian and upper Mississippian age sandstones, limestones and shales. Surface drainage from the facility flows to the south, eventually emptying into the east fork of the White River. ABG is approximately 20 acres near the east center boundary of NSWC Crane (Figure 8). It lies in Little Sulphur Creek Valley. Surface drainage flows into and from ABG via Little Sulphur Creek with the flow varying considerably with the seasons. Downstream from the center of ABG, surface flow ceases during the dry months as the water is captured by vertical infiltration into the sandstone and limestone aquifer underlying the area. Within ABG, there are designated areas for different methods of demilitarization including burn pads, burn pans, pink water tanks, incendiary cages and a primer pit. All current devices employed are equipped with run-on and run-off controls in the form of lids for pans or drains with sumps. Previous methods of demilitarization contributed to the soil and groundwater contamination.

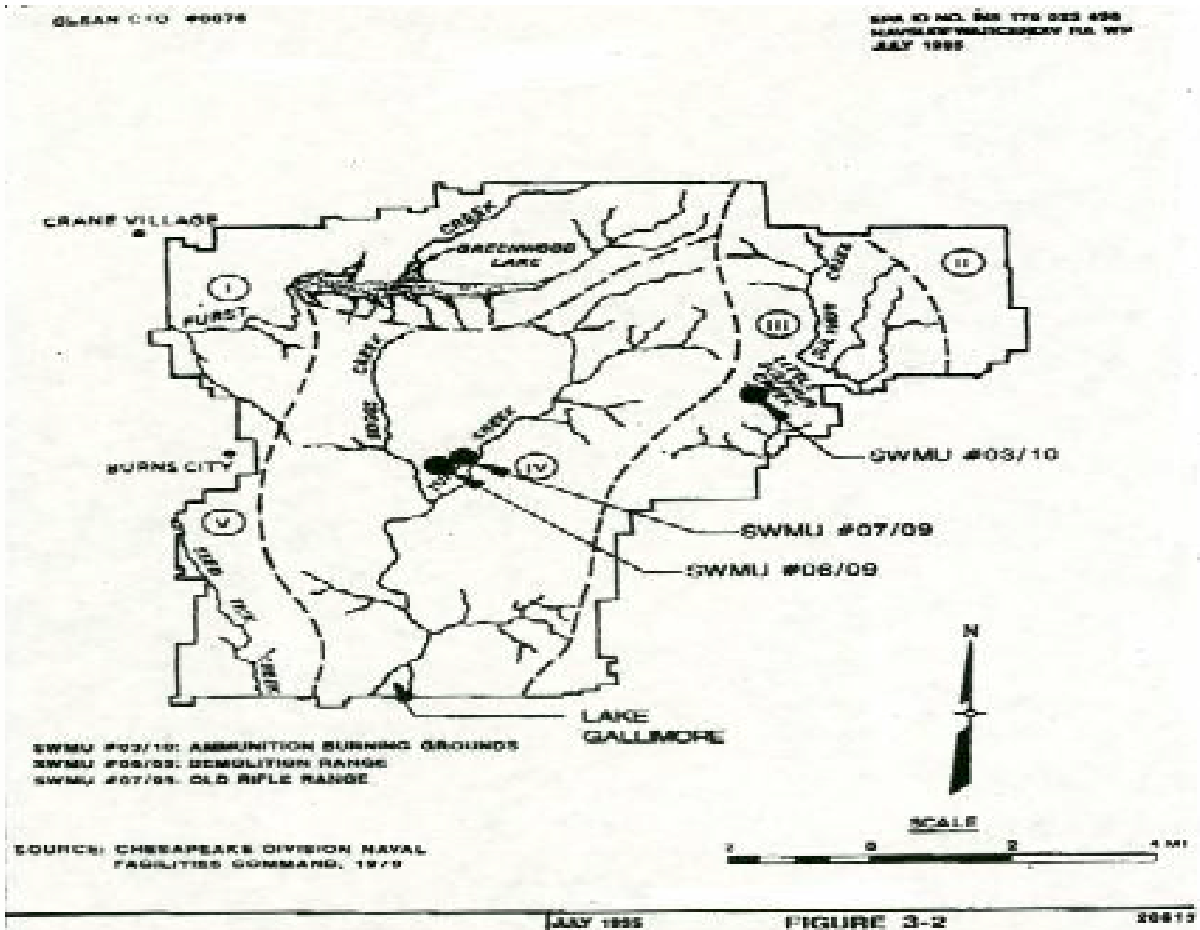

Figure 8. Map of Naval Surface Weapons Center - Crane, IN.

4.0 PERFORMANCE ASSESSMENT

There is not one clear-cut way to analyze the correctness of the results of the various assays for the detection of TNT and RDX. Several statistical methods were employed to evaluate the data from the field trials. One method compared the relative percent difference (RPD) between baseline concentration (Method 8330) and the result of the field screening method. The second method used linear regression curves of the field screening results versus Method 8330 concentrations. With this method, variations in the higher concentrations have a large effect on the regression line. The field results were also subjected to student's two-tailed paired t-test and Fisher F-test analysis. The paired t-test was used to determine if the differences between the sensors and the HPLC lab results were significantly different from zero with 95% confidence. The Fisher F-test was used to check for equality of variances. The bias and precision of each method was also evaluated for groundwater samples. Spikes of soil samples were not performed due to concern over accurate representation of spiked soil to weather-conditioned soil in regard to extraction efficiency and matrix effects. In addition to the statistical analysis, other factors were examined including false positives/negatives, analysis time, cross-reactants, analysis cost, sample size, use of solvents, and operator skill requirements.

4.1 LABORATORY STUDIES

The false positive/false negative rates were determined in water spikes as suggested by U.S. EPA Office of Solid Waste. Distilled water was spiked with either TNT or RDX at 0.5X and 2X the detection limit and analyzed. The goal is to obtain no response at the 0.5X level and 100% response at the 2X level. A false positive is a sample that gives a positive response below the stated detection limit while a false negative is one which does not generate a response above the detection limit. In addition to the spiked samples, the false positive/false negative rates were determined for the field groundwater and soil samples.

Bias, precision, method detection limit and reliable quantitation limits were determined in groundwater only. Method bias (accuracy) is determined with the following equation:

$$bias = (\overline{X} \ / \ X) * 100\% \qquad \text{Eq. 2}$$

where $\overline{x}$ is the mean value for seven or more replicate determinations and X is the spiked or characterized concentration. To determine the precision of the biosensor, the standard deviation and the mean are employed as follows:

$$\text{precision} = (s \ / \ \overline{X}) * 100\% \qquad \text{Eq. 3}$$

The U.S. EPA also requires the Method Detection Limit (MDL) and Reliable Quantitation Limit (RQL). (Ref. 20) The MDL is calculated from the low matrix spike standard deviation from the seven replicates:

$$\text{MDL} = 3.143\sigma \qquad \text{Eq. 4}$$

The RQL is four times the MDL.

Antibody cross-reactivity with compounds similar in structure were determined. The response of the antibodies to secondary targets is not equivalent or constant over concentration ranges for the secondary analyte. In a competitive immunoassay, an analyte (primary or secondary) causes a decrease in signal. The amount of cross-reactivity compound has with the antibody is reported as the concentration that causes a 50% decrease in signal or the IC_{50}. In a displacement assay, cross-reactivity is reported as the concentration of the secondary analyte needed to achieve a set response. This concentration is compared to the concentration of the primary analyte to achieve that same response.

4.2 RELATIVE PERCENT DIFFERENCE (RPD)

The RPD values between Method 8330 concentrations and the field screening results were calculated from equation 5 where D_1 = Field Screening concentration and D_2 = Method 8330 concentrations.

$$\mathbf{RPD = [(D1 - D2) / [(D1 + D2)/2]^{*} 100\%} \qquad \textbf{Eq. 5}$$

The smaller the RPD value, the closer are the concentrations of the two methods and the more accurate the field screening method. A positive RPD indicates that the field screening method gave higher concentrations than Method 8330 results. The reverse is true for a negative RPD. A value of ± 50 RPD is acceptable. (Ref. 18)

4.3 LINEAR REGRESSION

Linear regression plots were constructed to evaluate the accuracy of the field screening methods. The results from each method were plotted verses the Method 8330 results for each sample. A best-fit line was calculated for each assay method at each field test site. Under ideal conditions, true accuracy would have a slope = 1.0, y-intercept = zero, and a coefficient of determination (r^2) = 1.0. A slope greater than 1.0 indicates that the field screening methods generally give higher concentrations than Method 8330, and the reverse is true for slopes less than 1.0. The coefficient of determination indicates the amount of scatter in the data, with 1.0 indicating no scatter.

4.4 OTHER STATISTICAL VALUES

Other statistics used in the evaluation of the field data are the student's two-tailed, paired t-test and Fisher F-test on the raw data and t-test on the slope from linear regression analyses. The paired t-test indicates whether the immunosensor method gives significantly different analyte concentrations than the HPLC method, i.e., it is a test of accuracy. If the immunosensor is generating similar numbers as Method 8330, the result of the paired t-test will be that of no significant difference between the methods. The F-test assesses the variance of the data generated by the methods. In most cases, an accurate method will predict analyte concentrations that span the same range as those from the HPLC and there will be no significant difference between the variances. The t-tests on the slope from regression analyses determine whether or not these values differ significantly from zero. A slope

greater than zero indicates that the immunosensor method is capable of measuring varying levels of analyte concentrations.

From these properties, the following set of criteria was employed to assess the predictive capability of the immunosensor method for a given analyte at a particular site:

1. The student's paired t-test (95% confidence) result from the raw data must not be significant from Method 8330.

2. The F-test (95% confidence) result from the raw data must not be significant from Method 8330.

3. The slope must be positive and significantly different from zero as determined by t-test with 95% confidence.

Therefore, a method must satisfy all three criteria to be deemed predictive.

As mentioned earlier, these biosensor technologies are based on different principles and should be considered complimentary and not necessarily competitors. Due to these differences, the analysis of the FOB and the CFI will be discussed separately. Field demonstration results for both sensors will be compared to Method 8330 for TNT and RDX. Other factors used to evaluate the biosensors will also be examined.

4.5 FIBER OPTIC BIOSENSOR

Raw data from the field demonstrations and the laboratory analysis can be obtain from NRL. Since the geochemical conditions at each site are different, the analysis of the data is discussed separately for each location. All inhibition data were compared to standard curves to determine the concentration of the specific explosive. The TNT and RDX standard curves used for quantitation are shown in Figure 9.

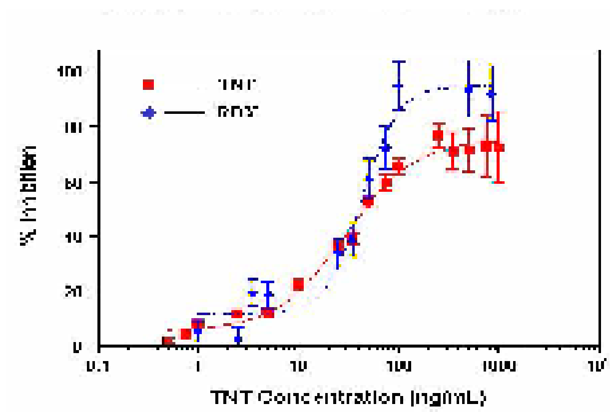

Figure 9. TNT and RDX Standard Curves.

4.5.1 False Positives/False Negatives Spikes

Following U.S. EPA protocols for false positive/negatives, buffer was spiked at 2X and 0.5X the MDL concentration. The MDL for the FOB for both RDX and TNT is 5 ppb, therefore the concentrations of the spikes tested were 2.5 and 10 ppb. The goal of any field analysis is to identify all samples containing RDX or TNT greater than the stated detection limit (i.e., no false negatives). At the higher concentration (10 ppb), there were no false negatives in either the RDX or TNT spiked samples (Table 2). Samples which do not contain explosives should also be accurately identified. With the lower concentration (2.5 ppb), there were 42% and 62% positives for RDX and TNT, respectively. The high level of positives at 2.5 μg/L can be partially explained by the standard curve and variability. The standard curves for RDX and TNT are asymmetric sigmoids which are linear in the middle range and gradually level off at the lower and upper ends of detection. This makes

it difficult to establish a precise limit of detection. If the cut-off for detection was exactly 5 μg/L, none of the 2.5 μg/L samples would have been positive.

Table 2. Fiber Optic Biosensor False Positives/False Negatives.

Sample	TNT MDL = (5 ppb)	RDX MDL = (5 ppb)
10 ppb RDX (20 replicates)	----	0% false negative
2.5 ppb RDX (20 replicates)	----	42% false positive
10 ppb TNT (20 replicates)	0% false negative	----
2.5 ppb TNT (20 replicates)	62% false positive	----

The variability between analyses can also affect the number of positives. With mass production of the antibody-coated fiber optic probes, there should be less variability due to improved QA/QC, therefore the MDL could be lowered to reduce the false positives without increasing the false negatives.

4.5.2 Cross-Reactivity (Water)

Both the limits of detection and the concentration at which 50% inhibition of the maximum signal (IC_{50}) occurred were determined for TNT and RDX (Table 3). Values greater than 1000 µg/L indicate no detectable inhibition. For the anti-TNT antibody from Strategic Diagnostics, only 1,3,5 trinitrobenzene (TNB) showed any appreciable level of cross-reactivity with detection at 10 μg/L and the IC_{50} at 50 μg/L (Table 3). Other compounds were detected with this antibody but did not achieve 50% inhibition of the signal for concentrations less than 1000 μg/L. This cross-reactivity to TNB is expected as the antibody was raised against a TNB conjugate. TNT could not be used because it is not immunogenic. There were no significant cross-reactants with the anti-RDX antibody at the IC_{50} level. Only HMX had any significant limit of detection with the anti-RDX antibody.

Table 3. Fiber Optic Biosensor Cross-Reactivity of Immobilized Anti-RDX and Anti-TNT Antibodies.

Sample	50%Inhibition (IC50) μg/L		Limit of Detection μg/L	
	RDX	TNT	RDX	TNT
RDX	33	> 1000	5	> 1000
HMX	> 1000	> 1000	100	> 1000
TNT	> 1000	46	> 1000	5
1,3,5-Trinitrobenzene	> 1000	500	1000	10
2-Amino-4,6-Dinitrotoluene	> 1000	1500	> 1000	50
2,4-Dinitrotoluene	> 1000	> 1500	> 1000	50-100
Tetryl	> 1000	> 1500	1000	150
1,3-Dinitroglycerin	> 1000	> 1000	1000	250
1,2-Dinitroglycerin	> 1000	> 1000	> 1000	350
4-Amino-2,6-Dinitrotoluene	> 1000	> 1500	1000	500
Dinitroethylene glycol	> 1000	> 1000	1000	500
1,3- Dinitrobenzene	> 1000	> 1500	1000	750
Trinitroglycerin	> 1000	> 1000	1000	> 1000
2,6-Dinitrotoluene	> 1000	> 1500	> 1000	1500
Nitrobenzene	> 1000	> 1500	> 1000	> 1500
2-Nitrotoluene	> 1000	> 1500	> 1000	> 1500
3-Nitrotoluene	> 1000	> 1500	> 1000	> 1500

Limit of Detection: lowest concentration to give more than 9% inhibition of the reference signal
IC_{50}: concentration that gives 50% inhibition of the reference signal

4.5.3 Matrix Effects (Groundwater)

The effect of different matrices on the explosive assays were examined by spiking each matrix with a high and low concentration of explosives. The results of this study are shown in Tables 4 and 5. The bias is the indication of how accurate the assay was (i.e., the similarity of the measured concentration to the spiked concentration). In all cases, the higher concentration was more accurate or had a bias closer to 100% (ideal) than the lower concentration. It should be noted that the % inhibition values were used to determine the bias and precision. The standard deviation from the % inhibition values was then converted to ppb to calculate the MDL and RQL values. The reason for this is the high TNT concentration is not on the linear portion of the standard curve. The inhibition values are at the level where dilutions should be performed to quantitate the sample. Very small changes have dramatic changes in the concentration values, which make the standard deviations

Table 4. Matrix Effects on TNT Fiber Optic Biosensor Assay.

Spike	Bias	Precision	MDL (ppb)	RQL
Umatilla Army Depot				
25 ppb TNT	77	12	4	16
250 ppb TNT	115	7	9	36
SUBASE Bangor				
25 ppb TNT	54	31	10	40
250 ppb TNT	77	12	11	44
LAAP				
25 ppb TNT	76	8	2	8
250 ppb TNT	97	8	9	36
Distilled Water				
25 ppb TNT	50	22	6	24
250 ppb TNT	91	11	12	48

Table 5. Matrix Effects on RDX Fiber Optic Biosensor Assay.

Spike	Bias	Precision	MDL (ppb)	RQL (ppb)
Umatilla Army Depot				
20 ppb RDX	38	41	10	40
75 ppb RDX	50	7	8	32
Crane NSWC				
20 ppb RDX	9	92	2	8
75 ppb RDX	87	10	9	36
LAAP				
20 ppb RDX	60	41	14	56
75 ppb RDX	83	6	3	12
Distilled Water				
20 ppb RDX	59	38	13	52
75 ppb RDX	90	9	8	32

very large. The TNT assay appears to have better values for the bias than the RDX assay. The precision varied in both assays but at the higher concentrations were less than 15%.

4.5.4 Field Standards (Groundwater)

Explosive standards were prepared by R. Araki of U.S. EPA Region 10 Manchester Laboratory for analysis during the initial field demonstration on SUBASE Bangor samples. The concentrations of TNT and RDX ranged from 1-5000 ppb (µg/L). Table 6 shows the results from the field analysis by the FOB and the Method 8330 laboratory results. The 1 ppb sample is below the detection limit of the biosensor. At the 10 ppb level, the biosensor was able to detect both RDX and TNT. It is noted that the concentrations determined by the fiber optic biosensor are lower than those obtained by Method 8330 direct injection. By employing an extraction to preconcentrate prior to Method 8330, the HPLC can detect lower levels. The higher concentrations of 1000 and 5000 ppb were above the percent inhibition levels that can be confidently used for accurate measurements. No dilutions were performed on the higher concentration samples to bring them down onto the curve. Table 6 gives the RPD's for the field standards with the averages being 37 and -13 for RDX and TNT respectively (Table 7). At lower detection levels, the RPD's are higher than the acceptable criteria of ±50[18,19] but as stated earlier, small variations at the lower concentrations greatly affect the RPD values.

Table 6. Fiber Optic Biosensor Field Standards at SUBASE Bangor.

	RDX			TNT		
Sample	**NRL Analyte 2000**	**QST Method 8330**	**RPD**	**NRL Analyte 2000**	**QST Method 8330**	**RPD**
FLS-1 (1 ppb TNT)	----	----	-----	2 ± 4	1	66
FLS-2 (10 ppb TNT)	----	----	-----	8 ± 4	10	-22
FLS-3 (100 ppb TNT)	----	----	----	38 ± 19	91	-82
FLS-4 (1000 ppb TNT)	----	----	-----	>200	960	
FLS-5 (5000 ppb TNT)	----	----	----	>200	5230	
FLS-6 (1 ppb RDX)	3 ± 7	1	93	-----	----	----
FLS-7 (10 ppb RDX)	11 ± 4	9	20	----	-----	----
FLS-8 (100 ppb RDX)	95 ± 41	97	-2	-----	----	----
FLS-9 (1000 ppb RDX)	>100	1110		----	----	----
FLS-10 (5000 ppb RDX)	>100	5220		----	----	----

Table 7. Fiber Optic Biosensor RPD Results for Field Samples.

Site	RDX		TNT	
	Avg RPD	RPD Range	Avg RPD	RPD Range
Standard Spikes	37	-2 to 93	-13	-82 to 66
SUBASE Bangor	19	-71 to 160	65	-40 to 198
Umatilla Army Depot	18	-67 to 188	78	-69 to 200
NSWC Crane	-92	-124 to -52		
Total Groundwater	-8	-124 to 188	74	-69 to 200
Soil	-7	-193 to 94	-38	-134 to 195

4.5.5 SUBASE Bangor (Groundwater)

The first field demonstration was performed on monitoring well and GAC effluent samples at SUBASE Bangor. During this demonstration, personnel from the U.S. EPA Region 10 and their contractors were trained to use the Analyte 2000 and the NRL fluidics unit. A summary of the results and the comparison to the independent QST laboratory's Method 8330 are shown in Table 8. Due to variations in fiber probe response and instrument noise (determined from blank samples), a conservative detection limit of 5 ppb was calculated from laboratory studies. Some fiber optic samples on Table 8 have concentration values listed lower than 5 ppb rather than below the detection limit (BDL) to give the full range of information on the sensor. The RPD's for RDX ranged from -71 to 160 with an average of 19 (Table 7). This average RPD value indicates that the fiber is slightly overestimating the RDX concentrations but is clearly within acceptable range. The samples with higher RPD's also were samples that had large standard deviations for the replicates. For TNT, the RPD's ranged from -40 to 198 with an average of 65 (Table 7). The positive RPD value indicates an overestimation of TNT concentration but the larger RPD's values are mostly associated with EW4, which has a value of 13 ppb. As with the RDX analysis, the higher RPD samples have the larger standard deviations for the % inhibition values.

Table 8. Fiber Optic Biosensor on SUBASE Bangor Samples.

Sample	RDX (ppb)			TNT (ppb)		
	NRL Analyte 2000	QST Method 8330	RPD	NRL Analyte 2000	QST Method 8330	RPD
INF1^{+}	29 ± 11	43	-39	BDL	2	
INF2	>200	455		BDL	2	
EW2	169 ± 185	356	-71	16 ± 11	24	-40
EW3^{+*}	33 ± 6	50	-41	16 ± 19	5	105

Table 8. Fiber Optic Biosensor on SUBASE Bangor Samples. (continued)

Sample	RDX (ppb)			TNT (ppb)		
	NRL Analyte 2000	QST Method 8330	RPD	NRL Analyte 2000	QST Method 8330	RPD
EW4+	27 ± 13	3	160	13 ± 11	0.1	198
EW5+	15 ± 2	19	-24	BDL	0.1	
EW6+	40 ± 7	42	-5	BDL	BDL	
EW7+*	106 ± 113	74	36	19 ± 10	20	-5
EW8	404 ± 453	562	-33	7 ± 14	BDL	
EW9+	10 ± 10	4	97	10 ± 13	BDL	
EW10+	299 ± 265	92	106	BDL	BDL	

+ Dilution performed to determine RDX values
* Dilution performed to determine TNT values
BDL - Below detection limit (MDL - 10 µg/L)

Another way to analyze the FOB data is to perform a linear regression on the data versus Method 8330. In this method, variations at the higher concentrations greatly affect the regression values for the slope. The linear regressions for RDX and TNT on SUBASE Bangor samples are shown in Table 9. The samples used for the regression analysis were ones in which both the FOB and Method 8330 gave numerical results. For RDX, the slope was 0.61 significantly different from 0 as determined with a t-test with 95% confidence with $r^2 = 0.67$. The TNT regression line (Figure 9) has a slope of 0.15 not significantly different from 0 as determined with a t-test with 95% confidence. The TNT results indicate that the FOB had no predictive value for the range of concentrations measured.

Table 9. Fiber Optic Biosensor Linear Regression Statistics.

Site	RDX			TNT		
	N	Slope	r^2	N	Slope	r^2
SUBASE Bangor	10	0.61	0.67	4	0.15	0.50
Umatilla Army Depot	19	0.51	0.40	10	0.31	0.25
NSWC Crane	9	0.42	0.84	----	—	---
Total Groundwater	38	0.61	0.65	14	0.37	0.28
Soil (µg/L)	12	1.13	0.87	12	0.88	0.92
Soil (mg/kg)	8	0.95	0.99	7	0.13	0.18

A student's two-tailed, paired t-test and the Fisher's F-test at 95% confidence levels was performed on the data with positive values in Table 8. The results are shown in Table 10. The FOB values for RDX passed both the t-test and the F-test in that neither was significant. The TNT values passed the paired t-test but were significant for the F-test with 95 % confidence (resulting $p<0.05$). It should be noted that the TNT analysis was on four samples with low levels of TNT and large standard deviations. This low number of degrees of freedom resulted in the strange outcome of the t-and F-tests. Usually a data set that passes the t-test will also pass the F-test, i.e. an accurate data set spans the same range as its reference. There were no false negatives for either RDX or TNT. The RDX assay had two false positives while TNT had four (Table 11).

Table 10. Fiber Optic Biosensor Paired t-test and F-test Results for Field Samples.

Site	RDX		TNT	
	Paired t-test (df)	F-test(df)	t-value (df)	F-test(df)
SUBASE Bangor	0.33 (9)	1.82 (9)	-0.75 (3)	22.13 (3)
Umatilla Army Depot	-0.19 (18)	1.52 (18)	-1.24 (9)	2.65 (9)
NSWC Crane	5.41 (8)	4.85 (8)	—	---
Total Groundwater	1.61 (37)	1.79 (37)	-1.37 (13)	2.09 (13)
Soil	-0.51 (11)	1.48 (11)	1.49 (11)	1.20 (11)

Table 11. Fiber Optic Biosensor False Positive/False Negative Results for Field Samples.

Site	RDX		TNT	
	FP	FN	FP	FN
SUBASE Bangor	2/11 (18 %)	0/11 (0 %)	3/11 (27 %)	0/11 (0 %)
Umatilla Army Depot	2/21 (10 %)	0/21 (0 %)	8/21 (38 %)	0/21 (0 %)
NSWC Crane	0/14 (0 %)	2/14 (14 %)	---	---
Total Groundwater	4/46 (9 %)	4/46 (9 %)	12/32 (38 %)	0/32 (0 %)
Soil	1/15 (7%)	2/15 (13%)	2/15 (13%)	0/15 (0%)

4.5.6 Umatilla Army Depot (Groundwater)

The second demonstration was on monitoring well and GAC effluent samples from Umatilla Army Depot. In the period between the two field trials, there was a major change in NRL personnel operating the FOBs. The U.S. EPA personnel and contractors remained the same. The summary of the data can be seen in Table 12. Twenty-one samples were analyzed at Umatilla with most of the

samples (17) requiring dilution to permit quantitation of either TNT, RDX or both. Dilutions at 1:10, 1:50 or 1:100 in water were performed on samples with % inhibitions greater than 70 and the diluted sample re-tested. The FOB and HPLC values of the diluted sample are given in Table 12 and used for all calculations. The RPD range for RDX is -67 to 188 and -69 to 200 for TNT (Table 7). The average RPD's are 18 and 78 for RDX and TNT, respectively. The average RDX RPD easily falls into the acceptable range of ± 50.

Table 12. Fiber Optic Biosensor on Umatilla Army Depot Samples.

Sample	RDX (ppb)			TNT (ppb)		
	NRL's Analyte 2000	QST Method 8330	RPD	NRL's Analyte 2000	QST Method 8330	RPD
WO22*	14 ± 15	14	0	12 ± 13	0.02	200
EW-1+	14 ± 4	9	43	>100	126	
WO-24+	9 ± 5	9	0	19 ± 11	BDL	
EW-4+	92 ± 94	20	129	BDL	0.45	
4-114	8 ± 9	16	-67	58 ± 43	94	-47
4-7+	15 ± 4	13	14	12 ± 12	BDL	
SB-3	9 ± 10	14	-43	BDL	BDL	
4-24	77 ± 18	39	66	BDL	BDL	
4-112*	21 ± 6	15	33	37 ± 9	16	79
4-102+*	31 ± 7	40	-25	18 ± 4	37	-69
EW-3+*	BDL	2		17 ± 16	8	72
4-117+	22 ± 11	21	5	59 ± 74	BDL	
4-3+	17 ± 4	13	27	BDL	0.1	
4-111+	BDL	BDL		76 ± 20	94	-21
4-25	27 ± 9	21	25	9 ± 8	BDL	
WO-21+	28 ± 6	39	-33	BDL	BDL	
009+*	9 ± 6	4	77	28 ± 11	23	18
4-113*	9 ± 14	9	0	BDL	1	
Combine 1+*	60 ± 12	118	-65	37 ± 26	3	172
Combine 2+*	72 ± 37	109	-41	67 ± 19	3	185
4-114D+*	11 ± 11	0.3	188	56 ± 15	2	187

+ Dilution performed to determine RDX values
* Dilution performed to determine TNT values
BDL - Below detection limit (MDL - 10 µg/L)

The linear regression result's for the Umatilla samples are shown in Table 9. The slope for the RDX regression is 0.51 with a coefficient of determination of 0.40. One sample (EW-4) seems to be associated with a high level of signal variation. This sample appears to have a significant effect on the r^2. The equation for the TNT linear regression is $y = 0.31x + 32.04$ with a $r^2 = 0.25$. The t-test on the slope indicates that it is not significantly different from zero at the 95% confidence level.

Statistical analysis of the Umatilla with a paired t-test and the F-test indicated that the FOB generated results for RDX and TNT that were not significantly different (at 95% confidence level) from Method 8330 (Table 10). As with Bangor, there were no false negatives for either RDX or TNT (Table 11). There were two false positives for RDX and eight for TNT. In several of the false positives, the cross-reactant TNB was present.

4.5.7 Naval Surface Weapons Crane (Groundwater)

The third field demonstration took place in September at the Naval Surface Weapons Center in Crane Indiana. At this site, there were problems with the assays, later identified in the laboratory as problems with the antibody-coated probes. Due to rapid degradation of TNT, we were unable to repeat the TNT analysis on the Crane samples in the laboratory. We were able to perform RDX analyses on the Crane samples back at NRL and the summary of the data is shown in Table 13. Only

Table 13. Fiber Optic Biosensor on NSWC Crane Samples.

Sample	RDX (ppb)		
	NRL Analyte 2000	QST Method 8330	RPD
Spring	36 ± 19	119	-107
03C03P2*	40 ± 9	68	-52
03C04	BDL	BDL	
10C55P2	12 ± 9	51	-124
10C55	84 ± 44	184	-75
10C57	BDL	BDL	
03C08AP2	57 ± 32	126	-75
03C10	50 ± 25	121	-83
03-34	BDL	41	
10-07	13 ± 6	29	-76
10-08	BDL	24	
10-17	9 ± 11	35	-118
10C37	BDL	BDL	
03C09P2	44 ± 9	146	-107

* Dilution performed to determine RDX values
BDL -Below Detection Limit (MDL - 10 µg/L)

one sample required dilution. The RPD’s ranged from -124 to -52 with an average of -92 (Table 7). This is out of the acceptable range and indicates underestimation of the concentration. This site has very different geochemistry from the other demonstration sites with acidic conditions and significant levels of trichloroethane. The RDX regression line y = 0.42x - 2.44 with a r^2 =0.84, indicating an underestimation of the concentration (Table 9). The slope passed the t-test with 95% confidence which denotes that the slope is significantly different from zero. The RDX data set from Crane did not pass either the student’s paired t-test ($p<0.05$) or the F-test($p<0.05$) at the 95% confidence level (Table 10). There were 2 false negatives but no false positives at Crane (Table 11).

4.5.8 Soil Field Samples

Ten archived, characterized soil samples (TJ00x) from several locations in the United States were provided by T. Jenkins of CRREL. In addition, H. Craig of U.S. EPA Region 10 provided us with five soil samples from Umatilla Army Depot, Hermiston OR. A summary of the soil extract results from the FOB and Method 8330 are shown in Table 14. It should be noted that a 1:20 dilution is always performed to get the proper acetone concentration in the test sample that is applied to the fiber optic biosensor. Because of this dilution, the MDL prior to dilution for a sample is 100 µg/L. Many of the samples required additional dilution to obtain quantitative values from the standard curve. Sample TJ005 extract, which was bright yellow, seemed to cause some problem with the fiber optic biosensor assay in that it gave values higher than the HPLC value, especially in the TNT assay. As it turns out, this sample contained high levels of picric acid which in its basic form is yellow. In an article by Zeck et. al. (Ref. 21), interference of the antibody-antigen interaction by picric acid is noted. Therefore, results from samples with picric acid should be examined further. The RPD values for RDX ranged from -193 to 94 with the average being -7 (Table 7). Ten samples had RPD’s less than ± 50. Only one sample (TJ005) seem to give an artificially high value which may be due to picric acid. The TNT assay did not perform as well as the RDX assay. The TNT RPD values ranged from -134 to 195 with an average of -38 (Table 7). Two of the samples gave RPD values less than ± 50 with five others in the ± 50-100 range.

Another approach for data analysis is to perform a linear regression on the fiber optic results versus Method 8330. The linear regressions results for RDX and TNT are shown in Table 9. The mean and standard deviation from seven or more analyses of each sample are shown. For RDX, the slope was 1.13 with an r^2 = 0.87 while the TNT assay gave values of slope =0.88 and r^2 = 0.92. Both slopes passed the t-test as being significantly different from zero at the 95% confidence levels. The RDX and TNT passed both the student paired t-test and the F-test by being not significantly different from Method 8330 with 95% confidence (Table 10). There was two false negatives and one false positive for RDX while there were no false negatives and two false positives for TNT (Table 11).

Table 14. Fiber Optic Biosensor on Soil Extract Samples.

Sample	RDX (µg/L)			TNT (µg/L)		
	NRL Analyte 2000	Method 8330	RPD	NRL Analyte 2000	Method 8330	RPD
TJ001	1100 ± 110	BDL		BDL	BDL	
TJ002	430 ± 74	352	20	350 ± 180	551	-44
TJ003	BDL	209		851000± 295000	915965	-7
TJ004	BDL	407		41800 ± 6600	49054	-16
TJ005	860 ± 70	50456	-193	102000 ± 11000	1205	195
TJ006	176000 ± 53100	147985	17	29400 ± 4500	82118	-95
TJ007	7200 ± 2900	8633	-18	98600 ± 16800	251548	-87
TJ008	116000 ± 10200	138500	-18	920 ± 60	BDL	
TJ009	550 ± 40	526	4	140 ± 30	BDL	
TJ010	2300 ± 400	2818	-20	3300 ± 270	434	154
G51-L1-A	2100 ± 100	2203	-5	900 ± 170	2660	-99
G16-L2-A	17500 ± 3300	14850	16	3300 ± 1400	12797	-118
G55-X-A	196000 ± 25000	135885	36	45800 ± 28600	231011	-134
G18-L3-A	8200 ± 2200	10259	-22	1100 ± 660	3698	-108
G18-L1-A	53900 ± 2100	19492	94	8400 ± 1990	23482	-95

BDL - Below detection limit(MDL - 10 µg/L)

For the T. Jenkins samples, we were supplied with the archived mg/kg values. In the CRREL Special Report 96-10, the authors reported each site has its own extraction efficiency but all were greater than or equal to 70% with the three minute acetone extraction method. (Ref. 20) Therefore, the µg/L concentration values were converted to mg/kg employing the assumption of 70% extraction efficiency . The results are shown in Table 15. Six of the eight RDX RPD values were ≤50%. The RPDs ranged from -85 to 156 with an average of 10. Again, the TNT results were not as clean. The TNT RPD values ranged from -154 to 197 (for TJ005) and an average of 10. Only one of seven TNT RPD's fall in the acceptable range. Table 9 shows the statistics for the linear regression of the calculated mg/kg FOB values for the samples supplied by T. Jenkins. The slope for the RDX assay is 0.95 with an r^2 of 0.99. The slope for TNT is 0.13 with an r^2 of 0.18. Since the extraction efficiency is not know for each sample, not further statistical analysis was performed on this data set.

Table 15. Fiber Optic Biosensor on Soil Samples.

Sample	RDX (mg/kg)			TNT (mg/kg)		
	NRL Analyte 2000	Method 8330*	RPD	NRL Analyte 2000	Method 8330*	RPD
TJ001	8 ± 1	1	156	BDL	BDL	
TJ002	3 ± 1	3	0	3± 1	4	-29
TJ003	BDL	4.4		6085 ± 2109	>750	
TJ004	BDL	BDL		299 ± 47	2318	-154
TJ005	6 ± 1	4	40	729 ± 79	6	197
TJ006	1258 ± 380	1247	1	210 ± 32	375	-56
TJ007	51 ± 21	127	-85	705 ± 120	1914	-92
TJ008	828 ± 73	986	-17	7 ± 0.4	4	33
TJ009	4 ± 0.3	4	0	1.0 ± 0.2	BDL	
TJ010	16 ± 3	19	-17	24 ± 2	2	169

*Values from T. Jenkins, CRREL
BDL - Below detection limit(MDL - 0.07 mg/kg)

4.5.9 Summary of Results

When the groundwater from all the sites is combined, the average RPD was -8 for RDX and 74 for TNT (Table 7). This suggests that in general the RDX assay is accurate. The RPD value for TNT is out of the acceptable range and indicates overestimation of the concentration. This may be due in part to cross-reactivity to TNB. When a linear regression in performed on the combined data set, the line for RDX is y = 0.61x +11.05 with an r^2 = 0.65 (Table 9). The slope is significantly different from zero (t-test with 95% confidence). For TNT, the slope of the regression line is 0.37 with an r^2 = 0.28 and is significantly different from zero. A student's two-tailed, paired t-test and a F-test with 95% confidence was performed on the combined data sets. Both the RDX and the TNT assay, showed no significant difference in either test (Table 10). In the combined data sets, there were two false negatives(9%) and four false positives (4%) for RDX while there were no false negatives and 12 false positives for TNT (Table 11).

As stated earlier, each assay must pass three criteria to be considered predictive. The three criteria are no significance (with 95% confidence) for the student's paired t-test and F-test and significant difference from zero for the linear regression slope. A summary of those results are shown in Table 16. For RDX, overall groundwater, Bangor groundwater, Umatilla groundwater, and soil passed all three criteria, therefore they were predictive. The RDX assay at Crane failed the t-test and F-test. The TNT assay passed the three criteria for overall groundwater and soil and are considered predictive for those tests. The TNT assay failed the F-test and the slope test on the four positive samples at Bangor. Only the slope test for TNT was failed at Umatilla. No TNT samples were

Table 16. Fiber Optic Biosensor Statistical Tests Summary.

Site	RDX				TNT			
	t-Test	F-Test	Slope test	Predictive	t-Test	F-Test	Slope test	Predictive
Groundwater (all)	Y	Y	Y	Y	Y	Y	Y	Y
Bangor	Y	Y	Y	Y	Y	N	N	N
Umatilla	Y	Y	Y	Y	Y	Y	N	N
Crane	N	N	Y	N	--	--	--	--
Soil	Y	Y	Y	Y	Y	Y	Y	Y

analyzed at Crane. From these tests, it appears the fiber optic biosensor can be predictive for RDX and TNT but there can be matrix interferences that would need to be addressed.

4.6 CONTINUOUS FLOW IMMUNOSENSOR

Raw data from the field demonstrations and the laboratory analysis can be obtained from NRL. As with the FOB, results are discussed per site.

4.6.1 False Positives/False Negatives Spikes

Experiments were conducted with the CFI to determine the false positive/ false negative percent for TNT and RDX. Explosive samples were prepared in the system flow buffer an into the CFI. Fluorescence dose responses were recorded from the immunosensor and calculated. The minimal detection limit with the FAST 2000 in the system flow buffer is 10 ng/mL. The false positive (FP)/ false negative (FN) experiments involved 20-30 replicate injections of TNT or RDX at concentrations at 5 ng/mL (FP) and 20 ng/mL (FN) into the CFI (Figures 10 and 11). The dotted line indicates the positive/negative cutoff line. Results showed 0% false positives and 0% false negatives (Table 17).

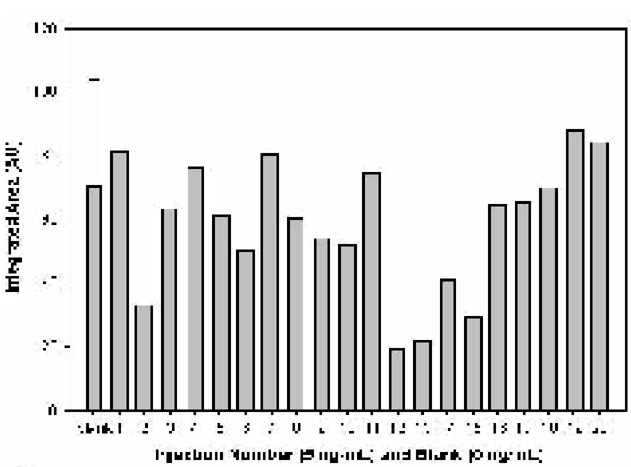

Figure 10. Replicate Injections of 5 ng/mL TNT (Test for False Positive Response).

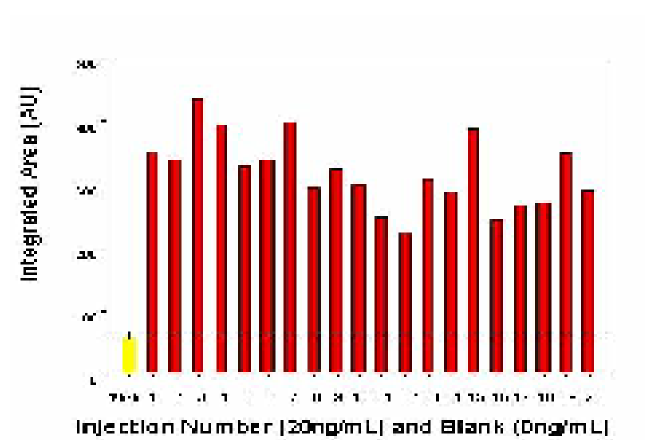

Figure 11. Replicate Injections of 20 ng/mL TNT (Test for False Negative Response).

Table 17. FAST 2000 False Positives/False Negative in Buffer.

Sample	TNT MDL=10ng/mL	RDX MDL= 10ng/mL
5ng/mL TNT (20 replicates)	0% positive	----
20ng/mL TNT (20 replicates)	0% negative	----
5ng/mL RDX (30 replicates)	----	0% negative
20ng/mL RDX (30 replicates)	----	0% positive

4.6.2 Accuracy and Precision (System Flow Buffer)

Two other performance criteria are accuracy and precision. Accuracy is an indication of how closely the average value of the CFI matches with the HPLC confirmatory method (SW846-Method 8330). Precision is an indication of how close the replicate injections into the CFI are to each other. Listed in Table 18 are results from the accuracy and precision experiments in which RDX and TNT (5 and 50 times the detection limit) in system flow buffer are injected into the CFI. Results indicate a high degree of accuracy between RDX and TNT with values that range from 93% - 99%. The precision of the sensor is also indicated, with percentages that are as low as 6% up to 15%.

Table 18. FAST 2000 Accuracy and Precision (System Flow Buffer).

Sample	TNT/RDX MDL=10ng/mL	
	Bias	Precision
50ng/mL TNT (9 replicates)	99	7
500ng/mL TNT (7 replicates)	93	14
50ng/mL RDX (7 replicates)	98	15
500ng/mL RDX (7 replicates)	99	6

4.6.3 Accuracy and Precision (Groundwater Matrix Spikes)

The groundwater spiked matrices give an indication of the environmental interferents that could pose problems for immunoassays. To determine the effect of groundwater matrixes on the analysis of TNT and RDX by the CFI, a series of experiments was performed. The first set of experiments required supplementing 3 different groundwater matrices (SUBASE Bangor, Umatilla Army Depot and Volunteer Army Ammunition Plant) with TNT and RDX at concentrations 5X and 50X the minimal detection limit. Each groundwater matrix selected contained little to no explosive content. Analysis by the CFI involved 7 injections of each spiked groundwater matrix onto the respective antibody/fluorescence antigen membrane complex. The fluorescence displacement area was recorded and translated into accuracy (%) and precision (%). Results indicated in Tables 19 and 20 show a wide percentage fluctuations for the matrix spikes in comparison to the system flow buffer data. TNT accuracy results ranged from 68% to as high as 653%. This high value (653%) can be

Table 19. Matrix Effects on RDX FAST 2000 Assay.

Spike	Bias	Precision	MDL (ppb)	RQL (ppb)
Umatilla Army Depot				
50 ppb RDX	20	37	7	28
500 ppb RDX	62	11	107	427
Bangor SUBBASE				
50 ppb RDX	55	9	7.5	30
500 ppb RDX	96	3	53	214
Volunteer, TN				
50 ppb RDX	N/D	N/D	N/D	N/D
500 ppb RDX	29	59	268	1074

Table 20. Matrix Effects on TNT FAST 2000 Assay.

Spike	Bias	Precision	MDL (ppb)	RQL (ppb)
Umatilla Army Depot				
50 ppb TNT	130	10	20	80
500 ppb TNT	97	86	409	1634
Bangor SUBBASE				
50 ppb TNT	212	26	85	340
500 ppb TNT	68	63	211	842
Volunteer, TN				
50 ppb TNT	653	41	475	1898
500 ppb TNT	142	15	324	1295

attributed to an interferent in the groundwater matrix that caused non-specific displacement of the fluorescence analog. This dramatic increase in fluorescence caused the data to be skewed on the higher end. Of the other matrix spikes, all were relatively accurate (within a factor of 2) in the measurement of TNT. Precision values were as low as 10% to as high as 86%. RDX accuracy measurements were not skewed as much as TNT. Using the EPA criteria, RDX percent accuracy ranged from 20% to as high a 96% while precision results ranged from 9% to 59%. Data calculations also reveal that the CFI was less affected by the matrix interferent at the higher concentrations than at the low end. Overall, the CFI was able to detect TNT and RDX with reasonable accuracy but did encounter matrix associated problems at each location. Elemental analyses of groundwater samples taken at one site (Volunteer Army Ammunition Plant) showed enormously high concentrations of sulfate, magnesium, carbon and alkalinity. These results suggest

that the CFI can provide a qualitative indication of explosive contaminants but, like most other immunoassay techniques, can encounter problems associated with the natural environment in quantitative determinations. Efforts to remove the environmental interferent by solid phase extraction are being investigated.

4.6.4 Cross-Reactivity (Groundwater)

Another performance criterion for the CFI is its ability to select and measure the unlabeled RDX or TNT molecule among other explosive compounds. To demonstrate the RDX immunosensors selectivity, a series of standard solutions containing various explosive compounds at 1000 ng/mL was injected into the immunosensor. As a calibrant, unlabeled RDX was also injected at the same concentration. After each injection of explosive samples, fluorescence integrated area from the displaced fluorescent RDX analog was recorded and compared to the fluorescence integrated area of the RDX standard (used as 100% value). Similar experiments were performed to determine TNT antibody cross-reactivity using 250 ng/mL as the explosive concentration. Exhibited in Table 21 are the percent cross-reactivity results of each explosive compound compared to unlabeled RDX and TNT measured by the CFI.

Table 21. FAST 2000 Cross-Reactivity of Anti-RDX and Anti-TNT Antibodies.

Sample	Anti-RDX Ab Cross-reactivity (%)	Anti-TNT Ab (11B3) Cross-reactivity (%)
RDX	100	1
2,4,6-Trinitrotoluene (TNT)	1.8	100
HMX	4.8	5
2-Nitrotoluene (NT)	1.9	9
3-Nitrotoluene	2.6	ND
4-Nitrotoluene	3.0	ND
Nitrobenzene (NB)	1.9	16
1,3-Dinitrobenzene (DNB)	2.8	ND
1,3,5- Trinitrobenzene (TNB)	3.8	600
Tetryl	0.95	38
2,4-Dinitrotoluene (DNT)	3.1	20
2,6-Dinitrotoluene	1.1	4
Trinitroglycerin	1.4	ND
2-Amino-4,6-DNT	1.3	21
4-Amino-2,6-DNT	1.8	1
1,2-Dinitroglycerin	1.8	ND
1,3-Dinitroglycerin	1.3	ND
Dinitro Ethylene Glycol	1.9	ND

ND - not determined

Results exhibited minimal cross-reactivity of other explosive compounds in the RDX assay. Percent cross-reactivity values ranged from as low as 0.9% (Tetryl) to 4.8% (HMX). The average percent cross-reactivity was approximately 2% for all compounds tested. One of the highest cross-reactivity values obtained was with HMX at 4.8%. It is reasonable to assume that the HMX molecule would exhibit high cross-reactivity results in the RDX immunoassay because of similar structural characteristics. TNT cross-reactivity experiments performed with the CFI involved injection of a series of standard solutions containing various explosive compounds at 250 ng/mL similar to that of the RDX immunoassay. As a calibrant, unlabeled TNT was also injected at the same concentration. Results shown in Table 21 show a 600% increase in cross-reactivity to trinitrobenzene (TNB). This is to be expected given the 11B3 anti-TNT antibody was raised against a TNB hapten complex. High cross-reactivity results of this nature can be positive given that many of the breakdown products of TNT are TNB and/or amino-DNT. Molecules such as TNB and its breakdown products, 2-amino-4,6-DNT, Tetryl and nitrobenzene are more cross-reactive than HMX or RDX because of their similar structural characteristics.

4.6.5 Field Standards (Groundwater)

The initial field demonstration conducted at SUBASE Bangor involved preparation and analysis of explosive standards (TNT and RDX). The explosive standards prepared at SUBASE Bangor served as calibrants while analyzing groundwater samples. Each explosive standard was analyzed by the CFI in a series of 7 injections (0.150 mL). A fluorescence peak area from the CFI was recorded for each injection. The explosives concentrations for each injection were calculated by comparing fluorescence peak areas of standards to samples, as described earlier. As seen in Table 22, RDX

Table 22. FAST 2000 Field Standards at SUBASE Bangor.

Sample	RDX			TNT		
	NRL FAST 2000	QST Method 8330	RPD	NRL FAST 2000	QST Method 8330	RPD
FLS-1	----	----	----	1 ± 0.1	1	0
FLS-2	----	----	----	15 ± 25	8	61
FLS-3	----	----	----	105 ± 53	91	14
FLS-4	----	----	----	965 ± 1102	960	1
FLS-5	----	----	----	4097 ± 1718	5230	-24
FLS-6	1 ± 0.3	1	0	----	----	----
FLS-7	8 ± 2	9	-12	----	----	----
FLS-8	113 ± 8	97	15	----	----	----
FLS-9	822 ± 77	1110	-30	----	----	----
FLS-10	3980 ± 390	5220	-27	----	----	----

standards analyzed by the CFI were consistent with the calculated value measured by QST Environmental Lab (e.g. FLS-8; 113 vs. 97). Standard deviations ranged from as low as 8% to as high as 31%. However, the highest standard deviation was only evident at the lowest concentration of 1.0 μg/L, where slight changes can skew standard deviation values. Calculated concentrations of TNT for the explosive standards (FLS-1 thru FLS-5) were also close to the expected values, determined by QST Laboratory. However, standard deviations were higher than expected, ranging from 16% to as high as 114% (FLS-4). A possible factor for the increased standard deviations is the low binding affinity of the anti-TNT antibody (11B3). Low affinity of the antibody to the explosive molecule, TNT, can result in fluorescence peak area differences seen even with multiple injections of the same standard solution.

Statistical calculations of the field data were performed as a measure of performance for the CFI. One such analysis performed was relative percent differences (RPD). In general, low RPD values (near zero) indicate the closeness of the two analytical methods (CFI verses HPLC). Calculated RPD's for the RDX and TNT field standards (Table 22) range from -30% to 15% and -24% to 61% (Table 23). For the RPD calculations only 1 sample was higher than ± 50 (FLS 2). The average RPD value of -11% and 10% for RDX and TNT is a good indication that the CFI was quite accurate in the determination of the explosive standards. However, a value as high as 50% seen in a standard could suggest a number of factors could be influencing the assay. Such factors could include fluorescence depletion on the membrane causing less displacement of fluorescence analog or variance in flow rates from instrument to instrument. Fluorescence depletion leading to decreased displacement efficiency will result in an underestimation of explosive standards and higher RPDs.

Table 23. FAST 2000 RPD Results for Field Samples.

Site	RDX		TNT	
	Avg RPD	RPD Range	Avg RPD	RPD Range
Standards	-11	-30 to 15	10	-24 to 61
SUBASE Bangor	-41	-146 to 60	118	-44 to 199
Umatilla Army Depot	-39	-165 to 87	-15	-185 to 197
NSWC Crane	-11	-168 to 107	83	-24 to 189
Total Groundwater	-31	-168 to 107	20	-185 to 197
Soil	-16	-195 to 122	86	-39 to 199

Linear regression analysis was also performed on the field standards. The RDX standards yielded a regression line of $y = 0.76x + 6.02$ with a $r^2 = 1.00$ (Table 24). The line for TNT was $y = 0.77x + 58.17$ with an $r^2 = 0.997$. The r2 values indicate a high level of precision between the CFI sensor and the HPLC method.

Table 24. CFI Linear Regression Statistics.

Site	RDX			TNT		
	N	Slope	r^2	N	Slope	r^2
Standards	5	0.76	1.00	5	0.77	0.997
SUBASE Bangor	11	0.67	0.48	7	1.58	0.96
Umatilla Army Depot	14	0.73	0.81	8	0.70	0.84
NSWC Crane	11	0.74	0.58	N/A	N/A	N/A
Total Groundwater	36	0.68	0.68	17	0.96	0.73
Soil (µg/L)	14	0.52	0.68	12	0.91	0.44

4.6.6 SUBASE Bangor (Groundwater)

At SUBASE Bangor, 13 groundwater samples were analyzed by the CFI for RDX and TNT content. Calculated concentrations of RDX determined by the CFI from samples, listed in Table 25, were within a factor of 2 of the value determined by QST Laboratory. Analyses of TNT content between the CFI and QST Laboratory were different. TNT concentration values listed in Table 25 revealed most of the samples were below the detection limit of the CFI, but did elicit a positive response (e.g., EW-9). Although the anti-TNT antibody (11B3) is specific for TNT it does exhibit minimal cross-reactivity to other compounds, which could result in an inaccurate response. As a result of the TNT data, further experiments were conducted to improve assay performance.

Table 25. FAST 2000 on SUBASE Bangor Samples.

Sample	RDX			TNT		
	NRL FAST 2000	QST Method 8330	RPD	NRL FAST 2000	QST Method 8330	RPD
EW-2	124 ± 9	356	-97	49 ± 9	24	68
EW-3	77 ± 8	496	-146	168 ± 36	263	-44
EW-4	64 ± 29	261	-121	57 ± 20	0.1	199
EW-5	345 ± 55	186	60	13 ± 6	0.1	197
EW-6	315 ± 204	419	-28	45 ± 18	BDL	
EW-7	68 ± 10	147	-74	1608 ± 304	977	49
EW-8	579 ± 100	562	3	79 ± 15	0.1	199
EW-9	799 ± 383	700	13	690 ± 428	BDL	
EW-10	478 ± 112	922	-63	39 ± 22	BDL	
INF 1	114 ± 41	429	-116	16 ± 3	2	158
BET 1	26 ± 16	7	115	BDL	BDL	
BET 2	10 ± 3	BDL		BDL	BDL	
EFF	BDL	BDL		91±12	BDL	

BDL - Below detection limit

RPD values were calculated for 13 groundwater samples containing TNT and RDX (Table 25). From these results, calculated RPD's were much higher than what is normally accepted. The average RPD for RDX and TNT were -41 and 118 with the values ranging from -146 to 60 and -44 to 199 (Table 23). RDX RPD values showed that seven out of the eleven samples with numerical values gave negative RPD values. Of those negative samples, three were above the -100% threshold (EW-3, EW-4 and INF-1) revealing a lowered estimation of RDX concentration by the CFI compared to the certified laboratory method (SW846-Method 8330). From the remaining four positive RPD values, only one (BET-1) was above the +100% threshold.

Another method for analyzing the field data is linear regression. The regression line for RDX is $y = 0.67x - 3.07$ with a $r^2 = 0.48$ (Table 24). TNT gave a line of $y = 1.58x - 1.54$ and a r^2 of 0.96. In RDX, the slope suggests an underestimation of the explosive while the reverse is true to TNT. Both the RDX and TNT assays passed the slope t-test by demonstrating slopes significantly different from zero with 95% confidence.

Statistical analysis of the RDX and TNT concentration data with the student's two-tailed paired t-test and the Fisher test with 95% confidence, gave results that indicated that the CFI data was not significantly different from Method 8330 (Table 26). Table 27 shows that there were no false negatives in either the RDX or TNT assay. There were two false positives for RDX and eight for TNT.

Table 26. FAST 2000 t-Test and F-Test Results for Field Samples.

Site	RDX		TNT	
	t-test (df)	**F-Test (df)**	**t-test (df)**	**F-Test (df)**
SUBASE Bangor	2.22 (10)	1.05 (10)	-1.14 (6)	2.59 (6)
Umatilla Army Depot	3.26 (13)	1.51 (13)	1.78 (7)	1.70 (7)
NSWC Crane	-0.18 (10)	1.06 (10)	-0.82 (1)	2.44 (1)
Total Groundwater	3.27 (35)	1.49 (35)	-0.04 (18)	1.27(16)
Soil	0.18 (13)	1.04 (13)	-1.94 (11)	1.91(11)

Table 27. FAST 2000 False Positive/False Negative Results for Field Samples.

Site	RDX		TNT	
	FP	**FN**	**FP**	**FN**
SUBASE Bangor	2/13 (15 %)	0/13 (0 %)	8/13 (62 %)	0/13 (0 %)
Umatilla Army Depot	0/20 (0 %)	5/20 (25 %)	4/20 (20 %)	4/20 (20 %)
NSWC Crane	1/15 (7 %)	1/15 (7 %)	2/14 (14 %)	1/14 (7 %)
Total Groundwater	3/48 (6 %)	6/48 (13 %)	14/47 (30 %)	5/47 (11 %)
Soil	1/15 (7%)	0/15 (0%)	3/15 (20%)	0/15 (0%)

4.6.7 Umatilla Army Depot (Groundwater)

Using lessons learned from SUBASE Bangor, the second series of field tests at Umatilla Army Depot showed significant improvements in the estimation of RDX and TNT by the CFI. The values listed in Table 28 for RDX concentrations determined by the CFI were in close proximity to that of QST laboratory except for two samples (4-102 and EW-1) that were considerably off. Determination of TNT concentration also improved on groundwater samples measured by the CFI. Groundwater samples measured by the CFI containing mid to high concentrations of TNT (e.g., Combo-2, 9, and 4-112) were accurately measured compared to those containing lower TNT concentrations (i.e., 4-113 and 4-114). This response could be due to a groundwater matrix interferent that can complex

Table 28. FAST 2000 on Umatilla Army Depot Samples.

Sample	RDX			TNT		
	NRL FAST2000	QST Method 8330	RPD	NRL FAST2000	QST Method 8330	RPD
4_3	59 ± 2	133	-37	BDL	0.1	
4_7	88 ± 31	132	-40	33 ± 19	BDL	
4_24	53 ± 18	39	30	BDL	BDL	
4_25	BDL	21		32 ± 6	BDL	
4_102	121 ± 24	402	-107	14 ± 3	367	-185
4_111	BDL	19		BDL	94	
4_112	39 ± 26	15	87	191 ± 18	164	15
4_113	BDL	9		BDL	63	
4_114	BDL	16		BDL	94	
4_114D	BDL	16		56 ± 11	94	-51
4_117	165 ± 53	209	-24	BDL	BDL	
9	77 ± 7	189	-84	958 ± 354	1160	-19
SB-3	BDL	14		48 ± 18	BDL	
WO-21	163 ± 14	389	-82	BDL	BDL	
WO-22	NA	14		NA	0.2	
WO-24	233 ± 22	470	-67	BDL	BDL	
EW-1	43 ± 28	450	-165	BDL	126	
EW-3	149 ± 51	112	28	457	846	-60
EW-4	902 ± 53	1020	-12	56 ± 30	0.4	197
Comb-1	607 ± 106	1180	-64	73 ± 28	138	-53
Comb-2	990 ± 101	1090	-10	190 ± 30	133	35

BDL- Below detection limit
NA - Not analyzed

with the explosive material, preventing recognition by antibody binding sites, causing no displacement of the fluorescent antigen.

Calculated RPD values (Table 28) show good correlation between the HPLC method performed by QST Laboratory and the CFI. The average RPD values for RDX and TNT were -39 and -15, with ranges of -165 to 87 and -185 to 197 respectively (Table 23). Of the 21 groundwater samples analyze for RDX, seven of the fourteen with numerical values above the detection limit were inside the acceptable ± 50% range. Only two samples (4-102 and EW-1) were above the -100% threshold. There were eight out of 21 groundwater samples analyzed for TNT that were above the MDL of the CFI and three of those samples were inside the acceptable ± 50 % range. Two of those nine samples gave values above the +100% threshold.

Linear regression plots for RDX and TNT at Umatilla Army Depot also indicated improvements in slope and r^2 for both analyzes (Table 24). For RDX, the regression line was y = 0.73x - 41.59 while the line for TNT was y = 0.70x - 4.70. The coefficient of determinations (r^2) were 0.81 and 0.84, respectively. Both assays passed the slope t-test at the 95% confidence level for being significantly different from zero. Even though there was improvement, the immunosensor still biased low on the explosive concentrations.

The RDX assay at Umatilla did not pass the two-tailed paired t-test with 95% confidence ($p<0.05$) but was found to be not significantly different with the F-test with 95% confidence (Table 26). The TNT passed both statistical tests. There were higher levels of false negatives for both RDX and TNT than had been previously observed at SUBASE Bangor (Table 27). There were five false negatives and no false positives for RDX, while there four false negatives and four false positives for TNT.

4.6.8 Naval Surface Weapons Center, Crane (Groundwater)

RDX and TNT analysis of 15 groundwater samples by the CFI at the Naval Surface weapons Center provided the most accurate and precise analysis of all the field demonstrations (Table 29). The "Spring" sample by Method 8330 gave a result that was right at or below the MDL set for the CFI. It was observed, particularly at the NSWC Crane, that the groundwater matrices can have a pronounced effect on the results when the explosive concentration is right at the detection limit of the instrument. Efforts to improve the TNT immunoassay were rewarded with most groundwater samples estimated by QST Laboratory being correctly estimated by the CFI. Most of the groundwater samples were low in TNT concentration or below the detectable limit of the CFI. The mean RDX RPD value for concentrations other than BDL was -11% (Table 23). The mean TNT RPD value of 83 was based on the only two values that were above the detection limit, and should not be considered a good indicator.

Linear regression plots for RDX at NSWC Crane show an r^2 of 0.58 (Table 24). The slope and the coefficient of determination for the TNT regression plot were not calculated due to the number of data points (2) not being statistically relevant. This low number of points is due to most of the samples being below the detection limit (BDL) and therefore, have no numerical value. The slope for the regression line were 0.74 for RDX. The slope for RDX is significantly different from zero, thereby passing the slope t-test. It should be noted that the RDX slopes for Umatilla, Crane and the field standards were 0.73, 0.73, and 0.76. The same slope range is seen with TNT at Umatilla and

the field standards (0.77 and 0.72). If this is a consistent trend, a correction factor could be employed to yield results very close to Method 8330.

Table 29. FAST 2000 on NSWC Crane Samples.

Sample	RDX			TNT		
	NRL FAST2000	QST Method 8330	RPD	NRL FAST2000	QST Method 8330	RPD
Spring	174 ± 78	119	38	115 ± 16	3	189
03C03	504 ± 35	678	-29	BDL	4	
03C04	BDL	BDL		BDL	BDL	
03C08	11 ± 5	126	-168	14 ± 9	BDL	
03C09P2	483 ± 62	146	107	BDL	BDL	
03C10	104 ± 41	121	-15	BDL	BDL	
03C12	17 ± 7	26	-40	BDL	BDL	
03_34	23 ± 10	41	-56	BDL	BDL	
10-07	54 ± 6	29	62	BDL	1	
10-08	BDL	24		BDL	1	
10-17	32 ± 16	35	-10	BDL	22	
10C37	BDL	BDL		BDL	BDL	
10C55	184 ± 56	184	0	40 ± 12	51	-24
10C55R	47 ± 18	51	-9	NA	BDL	
10C57R	66 ± 33	BDL		BDL	BDL	

BDL - Below detection limit
NA - Not analyzed

At Crane, the RDX assay passed both the student t-test and the Fisher test (Table 26) with 95% confidence. The results for the TNT assay (both tests were not significant) are suspect since the analysis was performed on only two positive samples. There was a single false negative each for RDX and TNT at Crane (Table 27). As for the false positives, there was one for the RDX assay and two for the TNT assay.

4.6.9 Soil Field Samples

The same samples as those described for the FOB were also analyzed by the CFI. It is important to keep in mind, that after the acetone extraction, 1.5 mL of the sample was dried down in a test tube with nitrogen and rehydrated with flow buffer for analysis. Table 30 shows the results from the CFI and Method 8330. As with the FOB, sample TJ005 caused some problems in the analysis for both

Table 30. FAST 2000 on Soil Extract Samples.

Sample	TDX (μg/L)			TNT (μg/L)		
	NRL FAST 2000	Method 8330	RPD	NRL FAST 2000	Method 8330	RPD
TJ001	400 ± 64	BDL		20 ± 6	BDL	
TJ002	530 ± 51	352	40	370 ± 110	551	-39
TJ003	60 ± 10	209	-109	1027000 ± 204000	915965	11
TJ004	40 ± 7	407	-167	482200 ± 117000	49054	163
TJ005	600 ± 120	50456	-195	342000 ± 115600	1205	199
TJ006	193400 ± 36100	147985	27	963000 ± 313000	82118	169
TJ007	8560 ± 920	8633	-1	183200 ± 48000	251548	-31
TJ008	92900 ± 6500	138500	-39	7300 ± 1020	BDL	
TJ009	370 ± 40	526	-36	14200 ± 1000	BDL	
TJ010	3470 ± 520	2818	21	87100 ± 25600	434	198
G51-L1-A	3550 ± 290	2203	47	5530 ± 1350	2660	70
G16-L2-A	36800 ± 3500	14850	85	27200 ± 16000	12797	72
G55-X-A	74400 ± 13000	135885	-58	219400 ± 67000	231011	-5
G18-L3-A	14355 ± 1440	10259	33	27900 ± 3300	3698	153
G18-L1-A	80500 ± 11400	19492	122	50600 ± 6300	23482	73

BDL -Below Detection Limit

the TNT and RDX assays. As stated earlier, results from samples with picric acid should be examined further. The RPD values for RDX ranged from - 195 to 122 with an average of -16 (Table 23). Eight of the fourteen values were less than ± 50. The TNT RPD values ranged from -39 to 199 with the average value of 86. Four of the samples were in the acceptable (± 50) RPD range. Several of the samples contained levels of TNB equivalent to or greater to those of TNT. As mentioned earlier, the 11B3 antibody is highly cross-reactive to TNB which may explain the high values for the CFI TNT assay. The linear regression values of the soil extracts are shown in Table 24. With the RDX analysis, TJ008 which is very high in HMX as well as TJ005 cause the linear regression to give a slope and r^2 (0.82 and 0.68). The equation for the TNT regression is $y = 0.91x + 164613.40$ with an $r^2 = 0.44$. The slope values passed the t-test for being significantly different from zero with 95% confidence.

Table 26 shows the values for the student's two-tailed, paired t-test and the Fisher test. Both RDX and TNT demonstrated no significant difference from Method 8330 at the 95% confidence level. No false negatives were found with the RDX soil assay but there was one false positive (Table 27). With the TNT soil assay, no false negatives were found but it did have three false positives. In one sample (TJ009) there was significant quantities of TNB in the absence of TNT. This would cause a response in the system, thereby generating a false positive. Cross-reactivity of HMX might also

be responsible for the TNT false positive for TJ008 as there is limited cross-reactivity with the 11B3 antibody for HMX. In general, there should be no matrix effects unless the cross-reactant or interferent is co-extracted into acetone.

As with the FOB, the values for the soil extracts was converted to mg/kg soil using a 70% extraction efficiency. The results are shown in Table 31. The RDX RPD's ranged from -161 to 100 with an average of -15 while the TNT RPD's ranged from -37 to 199 with an average of 90. Six out of nine of the RDX positive values and four of eight are in the acceptable RPD range. The linear regression analysis was also performed with the mg/kg values for the samples from T. Jenkins. The slope for RDX is 0.95 and the r^2 value is 0.94. The TNT assay gave a slope of 0.70 with an r^2 of 0.08. No further statistical analysis was performed on this converted data.

Table 31. FAST 2000 for Soil Samples.

Sample	RDX (mg/kg)			TNT (mg/kg)		
	NRL FAST 2000	Method 8330*	RPD	NRL FAST 2000	Method 8330*	RPD
TJ001	3 ± 1	1	100	0.1 ± 0.04	0.1	0
TJ002	4 ± 0.4	3	29	3 ± 1	4	-29
TJ003	0.4 ± 0.1	4.4	-161	7343 ± 1459	>750	
TJ004	0.3 ± 0.1	BDL		3448 ± 837	2318	39
TJ005	4 ± 1	4	0	2445 ± 827	6	199
TJ006	1383 ± 258	1247	10	6885 ± 2238	375	179
TJ007	61 ± 7	127	-70	1310 ± 343	1914	-37
TJ008	663 ± 93	986	-39	52 ± 7	4	171
TJ009	3 ± 0.3	4	-29	102 ± 7	BDL	
TJ010	25 ± 4	19	27	623 ± 183	2.0	199

*Values from T. Jenkins, CRREL
BDL - Below detection limit

4.6.10 Summary of Results

The average RPD was -31 for RDX and 20 for TNT for the combined groundwater data set (Table 23). This suggests that in general the RDX and TNT assays meet the performance objectives. When a linear regression in performed on the combined data set, the line for RDX is $y = 0.68x + 7.72$ with an $r^2 = 0.68$ (Table 24). The slope is significantly different from zero as determined by a t-test with 95% confidence. For TNT, the slope of the regression line is 0.96 with an $r^2 = 0.73$ (Table 24). A student's two-tailed, paired t-test and a F-test were performed on the combined data sets. The RDX assay passed the Fisher test with no significance at 95% confidence levels but failed the t-test with 95% confidence ($p<0.05$) (Table 26). The TNT assay showed no significant difference in either test.

In the combined data sets, there were three false negatives (6%) and six false positives (13%) for RDX while there were five (11%) false negatives and 14 false positives (30%) for TNT (Table 27).

In addition to the combined data, it is important to note that the groundwater slopes for Umatilla, Crane and the field standards range were 0.73, 0.73, and 0.76 for RDX. The same slope range is seen with TNT at Umatilla and the field standards (0.77 and 0.72). It is also important to the note the precision of this method especially with the field standards. If this is a consistent trend, a correction factor could be employed to yield results very close to Method 8330.

As stated earlier, each assay must pass three criteria to be considered predictive. The three criteria are no significance for the student's two-tailed, paired t-test and F-test and significant difference from zero for the linear regression slope, all with 95% confidence. A summary of those results are shown in Table 32. For RDX, Bangor groundwater, Crane groundwater, and soil passed all three criteria, therefore they were predictive. The RDX assay for overall groundwater and Umatilla groundwater failed the paired t-tests. The TNT assay passed the three criteria for overall groundwater, Bangor groundwater, Umatilla groundwater, and soil, therefore, they are considered predictive for those tests. From these tests, it appears the continuous flow immunosensor can be predictive for RDX and TNT but there can be matrix interferences that would need to be addressed.

Table 32. FAST 2000 Statistical Tests Summary.

Site	RDX				TNT			
	t-Test	F-Test	Slope test	Predictive	t-Test	F-Test	Slope test	Predictive
Groundwater (all)	N	Y	Y	N	Y	Y	Y	Y
Bangor	Y	Y	Y	Y	Y	Y	Y	Y
Umatilla	N	Y	Y	N	Y	Y	Y	Y
Crane	Y	Y	Y	Y	Y	Y	N	N
Soil	Y	Y	Y	Y	Y	Y	Y	Y

This page left blank intentionally.

5.0 COST ASSESSMENT

5.1 STARTUP COSTS

5.1.1 Fiber Optic Biosensor

Currently, the fiber optic device is commercially available (~$18K) but the antibody-coated optical probes are not. The uncoated fiber optic probes cost ~$15 each. The cost to coat the fiber probes with antibodies is largely based on personnel employed to perform the procedure. Currently at NRL, a single person can prepare 100 probes in a day and a half. Antibody-coated fibers may be stored more than 1 year lyophilized at ≤ 25°C or in buffer at 4°C. A computer (i.e., portable or laptop) is needed to operate the current fiber optic device via an RS232 port. NRL developed a semi-automated microfluidics unit for the addition of samples and reagents which is not commercially available but made from commercially available parts. The estimated cost of this unit is $8K.

In addition to the device and probes, there are some initial supplies (~$800) that are suggested but not required including adjustable pipettors and graduated cylinders. As the system becomes fully automated, the need for the pipettors will be eliminated. At the present time, a person with laboratory training is needed to operate the sensor but with automation this requirement will be diminished as will the labor costs. Up to 32 analyzes have been run on a single probe with each assay (sample, standard or reference) taking 12-17 minutes. The fiber optic biosensor can be battery operated or run off a line source (110V). It is recommended that the current biosensor be operated out of direct sunlight. Refrigeration of the stock solutions is the optimum storage condition but is not required. Stock solutions can be lyophilized for long term storage (± 1 yr) and rehydrated when needed with short term storage up to 1 month without refrigeration. The physical requirements pose minimal costs to the startup. Little, if any, cost is associated with site preparation and permits other than those to obtain the water samples.

5.1.2 Continuous Flow Immunosensor

The FAST 2000 was designed to be a field portable, single-channel instrument that uses a displacement immunoassay for detection of analytes. Currently, ten instruments have been produced by the manufacturer, Research International, at a cost of approximately $21,000/per instrument. The cost reflects the "custom" engineering of each instrument to date-- such factors as machining of individual parts, etc. Fluidics and hardware to maintain precise flow control during each analysis, software development costs are also involved.

Assay times are generally 2-4 minutes, allowing approximately 40-50 analyses per day. Set up and shut down can be completed in 15-20 minutes. Additional supplies required to run the instrument include the disposable coupons ($49/each) which are individually assembled and the antibody coated membranes/fluorescent analogs, prepared at NRL. RI is currently discussing several options for full-scale commercialization of the instrument, which would include injection molded coupons (reducing the cost to pennies per coupon), and membrane preparation by a company that currently sells immunoassay kits and produces TNT/RDX antibodies.

5.2 OPERATIONS AND MAINTENANCE

The consumables (buffers, pipet tips, syringes) are estimated to be $110/wk and are included in the $3-5/sample cost. Additional costs for acetone soil extractions are estimated at $1 - $1.50 per sample. Minimal training is required to operate the FOB. It can be run continuously or intermittently to allow for spot monitoring. For most groundwater monitoring during cleanup, intermittent (daily, weekly) monitoring is performed. The fiber optic sensor can be setup and assays run within an hour. Minimal waste is generated by the operation of the biosensor. Little maintenance of the Analyte 2000 has been required during the last four years of operation at NRL.

General operation of the FAST 2000 requires consumables similar to those needed for the Analyte 2000 (buffers, pipet tips, syringes, sample tubes), with an estimated cost of $3-5 per sample. Training of operators with technical backgrounds (engineers, environmental project managers) can be done in several hours. As discussed in the manual provided with the FAST 2000, maintenance of the fluidics in the instrument is essential to continued optimal performance. A shutdown routine is part of the software and provides an easy means of cleaning the instrument effectively after each use. More complete maintenance of the instrument to replace tubing or service the internal pump would require return of the instrument to the manufacturer, RI.

5.3 COST COMPARISONS TO CONVENTIONAL AND OTHER TECHNOLOGIES

Tables 33 and 34 on the following pages give a comparison of cost for the commercially available methods for explosive analysis in groundwater and soil, respectively (Refs. 19, 22). The initial set-up costs for the biosensors are high compared to the other technologies but the ongoing cost per sample is very low compared to the other methods. For a typical long-term groundwater remediation program, 50 to 150 samples will be tested per year (excluding quality assurance samples and individual extraction wells) for 10 to 30 years. Craig et al. estimated that after 500 samples, money is being saved by employing the biosensors versus the currently employed EnSys RIS method (Ref. 6). Both the EnSys RIS method and the NRL immunosensors currently require operation by personnel with some laboratory experience or with field analytical methods (Tables 35 and 36 on the following pages). The FAST 2000 is being automated so personnel with low skill level will be able to operate the instrument, thereby reducing labor costs.

Table 33. Technology Cost Comparison of Groundwater Explosive Analysis.

Method/Kit	On-Going Cost	Start-Up Costs	Training
Fiber Optic Biosensor	**$3-5/sample**	**Analyte 2000 $18K** **Fluidics unit - ~$8K**	**None**
Continuous Flow Immunosensor	$50 per coupon ~20-30 analysis per coupon or ~$3-5/sample	FAST 2000 $21K unit cost.	None
CRREL	$15/sample	$1500 for Hach spectrometer	None
EnSys RIS	$21/sample for TNT $25/sample for RDX $175/day or $450/week, $800/month for lab station	$1950 lab station cost	Training available. Applicable video on CRREL soil method available only
D TECH	$32.50/sample for TNT or RDX $300 for DTECHTOR (optional)		2 to 4 hours free on-site training
Ohmicron Rapid Assay	$13 to $20/sample, $175/day,$450/week or $800 for first month, $400 each additional month (rental)	$4000 for equipment	4 hours free on-site training
Method 8330	$200 - $1,000/sample depending on turnaround time	n/a	n/a

Table 34. Technology Cost Comparison of Soil Explosive Analysis.

Method / Kit	On-Going Costs	Start-Up Costs	Training
Fiber Optic Biosensor	$4-6.50/sample (includes extraction)	Analyte 2000 - $18K Fluidics Unit - ~$8K	None
Continuous Flow Immunosensor	$50 per coupon ~20-30 analysis per coupon or$4-6.50/sample (includes extraction)	FAST 2000 - $21K	None
CRREL	$15/sample	$1500 for Hach spectrophometer	Free video
Ensys RISc	TNT: $21/sample RDX: $25/sample	$1950 for lab station	Available- free
Dtech	$30/sample	$300 DTECHTOR (optional)	2-4 hrs free training
Idetek Quantix	$21/sample	$5880 for lab station	1 day free training
EnviroGard	Plate: $17/sample Tube: $20/sample	Plate: $4129 for equip and small supplies Tube: $2409 for equip. and small supplies	Available- free
Ohmicron RaPID Assay	$13-20/sample	$5500 for equip. purchase or rental for$800 1st month and $400 monthly thereafter	4 hrs free training

Table 35. Technology Comparison of Groundwater Explosive Analysis.

Method/Kit	Method Types and Analytes	Detection Range and Range Factor	Type of Results	Sample/Batch	Water Sample	Analysis Time	Skill Level
Continuous Flow Immunosensor	Immunosensor TNT, RDX, PETN	10-1000 ug/L	Quantitative	Sequential	150 uL /sample per injection.	3-4 min sample, plus 3-4 min internal standard 1 min peak analysis per sample	Medium/low
Fiber Optic Biosensor	Immunosensor TNT RDX	TNT:10-150 ug/L RDX:10-100 ug/L	Quantitative	Single up to a batch of 4	1.7 mL for 4 fiber analysis with fluidics unit	TNT: 8 min per quadruplicate sample or batch of 4 RDX: 16 min per quadruplicate sample or batch of 4 Double times to run reference analysis	Medium
CRREL	Colorimetric Ammonium Picrate/Picric Acid	AP/PA: 3.6 to 200ug/L (56X)	Quantitative	AP/PA: Single or batched	2L	20 minutes to hours to filter, faster per sample if batched; 20 minutes/sample to analyze	Medium/high
EnSys RIS	Colorimetric TNT, RDX and HMX Proposed Method 8510	TNT: 1 to 30 ug/L (30X) RDX: 5 to 150 (30X)	Quantitative	Single	2 L	20 minutes to a few hours for filtering TNT: 35 min/10 samples RDX: 50 min/sample	Medium
D-TECH	Immunoassay - ELISA TNT RDX	TNT and RDX: 5 to 45 ug/L (9X) with DETECHTOR TNT and RDX: 5 to 60 ug/L (12X)	Semiquanti-tative (concentra-tion range)	8 (single or batch)?	1 mL	40 minutes for 8 samples for TNT and RDX 10 to 15 minutes for single sample	Low
Ohmicron RaPID Assay	Immunoassay - ELISA Magnetic particle/tube kit TNT	TNT: 0.07 to 5 ug/L (71 X)	Quantitative	10 to 40 (batch only)	100 uL	70 minutes for 10 samples	High, initial training recommended
Method 8330	High Performance Liquid Chromatography	Direct injection: RDX : 14 ug/L TNT : 7 ug/L Salting out and extraction: RDX: 0.84 ug/L TNT: 0.11 ug/L	Quantitative	Single	100 uL	20 min/sample If<20 ug/L need salting - out extraction ~2-3 hours/sample	high

Table 36. Technology Comparison of Soil Explosive Analysis.

Method / kit	Method type/ analytes	Detection range	Type of result	Samples/ batch	Sampl e size	Sample preparation time	Sample analysis time	Skill level required
Fiber Optic Biosensor	Immunoassay TNT, RDX	TNT: 0.7-21 mg/kg RDX: 0.7-14 mg/kg	quantitative	1-4	5 g	3 min shaking in 25 mL acetone, settle	TNT: 8 min per quadruplicate sample or batch of 4 RDX: 16 min per quadruplicate sample or batch of 4 Double times to run reference analysis	Medium
Continuous Flow Immunosensor	Immunoassay TNT, RDX	0.05 - 5 mg/kg	quantitative	1	5 g	3 min shaking in 25 mL acetone, settle	3-4 min sample, plus 3-4 min internal standard 1 min peak analysis per sample	Medium/ low
CRREL	Colorimetric TNT,RDX, 2,4DNT, ammonium picrate, picric acid	TNT: 1-22 mg/kg RDX: 1-20 mg/kg	quantitative	TNT: batch or single RDX: 6-7	20 g	3 min shaking in 100 mL acetone, filter	5 min/ sample	Medium
Ensys RISc	Colorimetric TNT,RDX	TNT, RDX: 1-30 mg/kg	quantitative	single	10 g	3 min shaking in 50 mL acetone, 5 min to settle, filter	40 min per 10 samples	TNT: low RDX: Medium
Dtech	Immunoassay TNT,RDX	TNT:0.5-5.0 mg/kg	semi-quantitative (concentration range)	4 single or batch	3 ml ~4.5g	3 min shaking in 6.5 mL acetone, 1-10 min to settle	30 min per 1-4 samples	Low
Idetek Quantix	Immunoassay TNT	TNT; 0.25-100 mg/kg	quantitative	20-40 batch only	~4.2 g	3 min shaking in 21 mL acetone, settle	2.5-3.5 hours for 20-40 samples	Medium-High
EnviroGard	Immunoassay TNT,RDX	Plate: TNT, RDX 1-100 mg/kg Tube: TNT, RDX 0.2-15 mg/kg	Plate: quantitative Tube: semi-quantitative	Plate: 8 per batch Tube: 14 per batch	2 g	Air dry soil, 2 min shaking in 8 mL acetone, filter	Plate: 90 min for 8 samples Tube: 30 min for 14 samples	Plate: Medium-High Tube: Medium
Ohmicron RaPID Assay	Immunoassay TNT,RDX	TNT: 0.07-5 mg/kg	quantitative	5-51 batch only	10 g	1 min shaking in 20 mL methanol, 5 min to settle, filter	1 hour for 20 extractions; 45 minutes for analysis (51 samples)	Medium-High

6.0 IMPLEMENTATION ISSUES

6.1 COST OBSERVATIONS

The Analyte 2000 and the FAST 2000 are commercially available from Research International. The cost of the instruments is determined by RI but it should be kept in mind that <15 of either instrument have been manufactured to date. On the other hand, the antibody-coated surfaces (probes or membranes) currently are not commercially available. This work is still being done at NRL, partly due the availability of the antibodies. RI is currently investigating methods to commercialize these items. As with the instruments, mass production of these items should have a positive effect on their costs. Each CFI coupon is currently out of polycarbonate, but has been designed with the goal of injection molding for field use.

6.2 PERFORMANCE OBSERVATIONS

As mentioned earlier, matrix effects were observed with both immunosensors. In addition to filtering to remove large particles, it is recommended that the standards used in each method be made up using blank groundwater from that site. Over the course of the field trials, improvements in the analysis were observed as the operators became more familiar with the instrument responses. They could easily identify potential problems from the raw data rather than after data calculations.

With the fiber optic biosensor, variability especially between probes is an issue. Mass production of the antibody-coated probes, with appropriate QA/QC, versus the small batches prepared in our laboratory, should reduce some of the variability noted in this study. This progress may also improve the limit of detection.

6.3 OTHER SIGNIFICANT OBSERVATIONS

Research International has licensed key patents related to the fiber optic biosensor. Current focus of this technology has been on the development of a fully automated system for the U.S. Marines and Special Forces for the detection of biological warfare (BW) agents (proteins, toxins, bacteria, etc.). DARPA and ONR have jointly funded a Phase II SBIR to produce inexpensive, manufacturable fiber optic probes for the biosensor. SERDP has funded a project for the proof of principle for deployment of the fiber optic biosensor into a cone penetrometer for detection of explosives. In addition to the BW and explosive applications, collaborations with NSWC Carderock are adapting the Analyte 2000 for the detection of polyaromatic hydrocarbons. Assays for the rapid detection of sepsis markers with the Analyte 2000 are being pursued in a collaboration with WRAIR and AGEN Biomedical, LTD. The market for a fast, sensitive sepsis test includes not only medical diagnostics and casualty care but also food processing and beverage production.

The FAST 2000 is currently being commercialized by Research International. The company has licensed the NRL patent for the technology, has sold several instruments to the U.S. EPA, and is actively pursuing marketing partners and possible market niches. RI is working with NRL to solve the problems identified with instrument reliability. To overcome problems with matrix effects at the low ends of detection, the U.S. EPA is providing additional samples for screening. These field

samples will be prepared as before, with simple buffer addition, and will also be pre-treated using a solid phase extraction protocol.

The most effective pathway for transferring this technology is through the current FAST 2000 manufacturer, Research International. The company has built 10 instruments, has actively exhibited the instrument at major trade shows, has indicated its commitment to commercializing the technology by signing a licensing agreement with NRL and is actively holding talks with several larger companies that would serve as marketing/development partners. The manufacturer has been involved with technical assistance and instrument maintenance throughout this process. The company has made modifications as required to improve field trial performance.

6.4 REGULATORY AND OTHER ISSUES

The field trial results have been incorporated into submissions to the U.S. EPA Office of Solid Waste with the goal of obtaining a method number under OSW 846. To guide our efforts, we have had ongoing conversations with Barry Lesnick at the U.S. EPA. Examination of the validation data by Barry Lesnick and the working group was positive. The methods are undergoing final edits before being published as draft methods. The next biggest hurdle will be encouraging use of the methods by site managers.

6.5 LESSONS LEARNED

6.5.1 Groundwater

Several lessons were learned regarding the fiber optic biosensor during these field trials. The main lesson was the need to make sure the QA/QC procedures for the preparation of the antibody-coated fiber optic probes are clearly stated and emphasized to all. This is especially true with new personnel. Another point that needed to be addressed was determining when the fiber optic probe was no longer useable for data analysis. Several times at the first field demonstration, analyses of samples were performed on fiber probes that were no longer functioning optimally. From these trials, it was noted that possible instrument problems (symptom and possible cause) that may occur should be written out for the operator. This is important as several of the Analyte 2000s, which have been use heavily for 3-4 years, are now hitting their lifetime. Variability in laser power is one problem in older, heavily utilized instruments. Overall, the Analyte 2000 is a durable instrument but all things have a limited lifetime. Fluidics problems such as clogging, leaks, etc., are something that any instrument working with real world samples will have to address. The symptoms, possible causes, and solutions need to be stressed to the operator so time, reagents, and samples are not wasted.

In addition to issues with the instruments, matrix effects were observed with the fiber optic biosensor. The importance of filtering the samples was clearly demonstrated in the field, as the presence of particulates and/or cloudiness was observed in many samples. The standard curves which are used for quantitation are created from explosive spikes into distilled water. From these studies, we now recommend that the standard curves should be created with explosive spikes into blank water from the test site to reduce or eliminate matrix effects.

For the flow immunosensor, early results with the FAST 2000 varied widely, most probably due to the nature of the dose/response curve of the analysis. At the conclusion of field trial 1 (SUBASE Bangor), an improvement was implemented in the immunoassay protocol, i.e., the insertion of more internal standards during the 7 injections of each groundwater sample to achieve a closer approximation of the unknown concentration. In addition, the choice of standard is critical for quantitation. The standard should give a similar fluorescence increase as the test sample. These modifications to protocols and recognition of constant fluorescence depletion of the membrane with time proved important for later accuracy and precision measurements by the FAST 2000 immunosensor. In addition, some technical expertise with other EPA methods would, in our opinion, be necessary to understand and fully use the instrument as is. This is primarily due to the complex nature of the response of the instrument to the analyte over the time of the instrument usage.

Additional lessons were gleaned from the studies on matrix interferences. Several samples varied widely even after filtering. Matrix effects are of major concern, since especially high salt concentrations or other compounds may interfere with antibody selectivity and binding or quench fluorescence. In a few of the sample matrixes, a slight change in the background signal just before the sample signal on the continuous flow sensor was observed. This was usually observed as a decrease in the background signal, but in a few cases, this developed as a slight increase or a spike above background. Part of this was due to our using highly purified water for the standards, which differs in its components from the matrixes of the sample. Making the standards in a blank water sample acquired from the site that is being monitored could normalize matrix effects. The matrix effects will still be present but will essentially be subtracted out after all the calculations have been performed. In any event, we did not observe the matrix ever masking the signal of even the lowest standards tested.

As seen in the results, we found that differing hydro chemistries at each site affected final determinations of TNT/RDX. For the flow immunosensor, samples are generally tested without dilutions, extractions or selective prefiltration. Therefore, it is recommended that matrix spikes be analyzed prior to running actual samples. To run matrix spikes, “blank” groundwater from the remediation site (defined as having TNT and RDX concentrations below the MDL of Method 8330) is spiked with TNT/RDX concentrations 5 and 50 times the MDL of the CFI (i.e., 10 ng/mL). Replicates of these matrix spikes (50 and 500 ng/mL final concentration) are then tested in the CFI and compared to standards of identical concentrations. These changes are incorporated into the SOP’s. In summary, we determined the importance of setting up instrument calibrations that were specific for each sites.

It became clear with both systems that further studies into sample preparation to prevent matrix effects would greatly improve the accuracy and precision of the sensors. Solid phase extraction is preferred method used to reduce matrix contaminants which effect the assays and to improve detection limits by preconcentrating the sample. In limited laboratory tests performed using SPE samples, we found it to be an effective way to improve overall assay reliability. SPE is also recommended for those sites where samples are at the lower end of the method detection limit.

6.5.2 Soil

The analysis of soil samples for TNT and RDX requires an extraction of the explosive material from the soil. For this study, we utilized a field method of extraction developed by Tom Jenkins (CRREL) that can be performed in less than five minutes. The method dictates that 20g of soil be mixed with 100 mLs of acetone, in a certified clean vial, and shaken for 3 minutes. The mixture then sits for a short period of time to allow the particulates to fall out of suspension or the extract may be filtered. The fiber optic biosensor uses the acetone extract directly, replacing the 5% acetone in the sample fluorescent solution, thereby performing a 1:20 dilution. This raises the limit of detection by 20. In the continuous flow immunosensor method, sample preparation involves placing 2 mLs of the extraction supernatant into a test tube and removing the acetone using an argon stream. The remaining material is then brought up in 2 mLs of the assay buffer. Direct injection of the prepared sample and subsequent analysis allow for semi-quantitative analysis of the soil.

Similar to lessons learned with groundwater, we found that the highly heterogeneous nature of soils can lead to a high degree of variability in the amount of explosives material found in the extract. Also, by using a strongly polar solvent, like acetone, to perform an extraction, a wide variety of other materials will be contained in the sample that may cause anomalies during analysis. The nature of these matrix-related effects are not specifically known, but they do appear to be ubiquitous in soils collected from many different sites. In several cases, the HPLC analysis showed high levels of cross-reacting species, further emphasizing the importance of a complete site characterization prior to implementing any routine monitoring program. As with the groundwater, these effects can be mitigated by further treatment of the acetone extract using SPE protocols. The additional work required to perform the SPE adds significantly to the time and cost of sample preparation. However, samples prepared using SPE provide improved accuracy and precision of the assay.

7.0 REFERENCES

Key

1. L.C. Shriver-Lake, N.A. Naz and F.S. Ligler, "A fiber optic biosensor for the detection of TNT/RDX in environmental samples", Current Protocols in Field Analytical Chemistry (ed. V. Lopez-Avila), John Wiley & Sons, 1998, 1E1-12.

2. Bart, John C., L.L. Judd, K.E. Hoffman, A.M. Wilkins, P.T. Charles, and A.W. Kusterbeck "Application of a portable immunosensor to detect explosives TNT and RDX in groundwater samples" Environmental Science and Technology, 1997, **31(5)**, 1505-1511.

3. Crockett, A.B., H.D. Craig, and T.F. Jenkins. "Field Sampling and Selecting On-Site Analytical Methods for Explosives in Water", Federal Facilities Forum Issue, EPA/600/S-99/002, 1999, U.S. Environmental Protection Agency.

4. Crockett, A.B., T.F Jenkins, H.D. Craig, and W. E. Sisk (1998) "Overview of on-site analytical methods for explosives in soil" Cold Regions Research and Engineering Laboratory (CRREL) Special Report 98-4.

DoD contracts/records

5. ESTCP Final Report: Original records stored at Code 6900, Naval Research Laboratory, Washington, DC 20375.

General

6. J.P. Golden, L.C. Shriver-Lake, G.P. Anderson, R.B. Thompson, and F. S. Ligler "Fluorimeter and tapered fiber optic probes for sensing in the evanescent wave" *Optical Engineering*, 1992, **31(7)**, 1458-1462.

7. F.S. Ligler, J.P. Golden, L.C. Shriver-Lake, R.A. Ogert, D. Wijesuria, and G.P. Anderson "Fiber optic biosensor for the detection of hazardous materials" *Immunomethods*, 1993, **3(2)**, 122-127.

8. G.P. Anderson, J.P. Golden, L.K. Cao, D. Wijesuria, L.C. Shriver-Lake, and F.S. Ligler "The development of an evanescent wave fiber optic biosensor" *IEEE Engineering in Medicine and Biology*, 1994, **13(3)**, 358-363.

9. L.C. Shriver-Lake, K.A. Breslin, J.P. Golden, and F.S. Ligler "Detection of TNT using an evanescent wave fiber optic biosensor" *Analytical Chemistry*, 1995, **67(14)**, 2431-2435.

10. M.D. Pease, L.C. Shriver-Lake, and F.S. Ligler "Adaptation of a fiber-optic biosensor for use in environmental monitoring" in *Biosensor and Chemical Sensor Technology*, ACS Chemical Society, 1995, 33-43.

11. Craig, H., G. Furguson, A. Markos, A. Kusterbeck, L. Shriver-Lake, T. Jenkins, and P. Thorne (1996) Field demonstration of On-Site Analytical Methods for TNT and RDX in Groundwater. Proceedings of the Great Plains - Rocky Mountain Hazardous Substance Research Center (HSRC)/Waste Education and Research Consortium (WERC) Joint Conference on the Environment, Alberquerque, NM, May 21-23, l996, pp 204-219.

12. L. C. Shriver-Lake, B.L. Donner, and F.S. Ligler, "On-site monitoring of TNT with a portable fiber optic biosensor" *Environmental Science and Technology,* 1997, **31(3)**, 837-841.

13. B.L. Donner, L.C. Shriver-Lake, and F.S. Ligler, "Transition from laboratory to on-site environmental monitoring of TNT using a portable fiber optic biosensor" in *Immunochemical Technology for Environmental Applications*, ACS Symposium Series 657, (D. Aga and E. Thurman, eds.), American Chemical Society, Washington DC (1997) 198-209.

14. L.C. Shriver-Lake, N.A. Naz and F.S. Ligler, (1998) "A fiber optic biosensor for the detection of TNT/RDX in environmental samples", Current Protocols in Field Analytical Chemistry (ed. V. Lopez-Avila), John Wiley & Sons, 1E1-12.

15. J.P. Golden, E.W. Saaski, L.C. Shriver-Lake, G.P. Anderson, and F.S. Ligler "A portable multichannel fiber optic biosensor for field detection" *Optical Engineering*, 1997, **36(4)**, 1008-1013.

16. J.P. Golden, G.P. Anderson, S.Y. Rabbany, and F.S. Ligler "An evanescent wave biosensor - part II: Fluorescent signal acquisition from tapered fiber optic probes" *IEEE Transactions Biomedical Engineering*, 1994, **41(6)**, 585-591.

17. J.P. Whelan, A.W. Kusterbeck, G.A. Wemhoff, R. Bredehorst, and F.S. Ligler "Continuous-flow immunosensor for detection of explosives" *Analytical Chemistry*, 1993, **65,** 3561-3565.

18. L.L. Judd, A.W. Kusterbeck, D.W. Conrad, H.Yu, H.L. Myles Jr., and F.S. Ligler "An antibody-based fluorometric assay for detection of the explosives TNT and PETN" *SPIE*, 1995, **Vol 2388**, 198-204.

19. J.C. Bart, L.L. Judd, K.E. Hoffman, A.M. Wilkins, P.T. Charles, and A.W. Kusterbeck : "Detection and Quantitation of the explosives TNT and RDX in groundwater using a continuous flow Immunosensor" in Immunochemical Technology for Environmental Applications, ACS Symposium Series 657, eds: D. Aga and E. Thurman, American Chemical Society, Washington DC, 1997, 210-220.

20. Bart, John C., L.L. Judd, K.E. Hoffman, A.M. Wilkins, P.T. Charles, and A.W. Kusterbeck "Application of a portable immunosensor to detect explosives TNT and RDX in groundwater samples" Environmental Science and Technology, 1997, 31(5), 1505-1511.

21. Bart, John C., L.L. Judd, and A.W. Kusterbeck "Environmental immunosensors for the explosive RDX using a fluorescent dye-labeled antigen and the continuous flow immunosensor" 1997, Sensors and Actuators B, 1997, 38-39, 411-418.

22. Kusterbeck, A.W., P.R. Gauger, and P.T. Charles "Portable flow immunosensor for detecting drugs and explosives" SPIE, 1997, Vol 2937, 191-196.

23. Crockett, A.B., H.D. Craig, and T.F. Jenkins. Field Sampling and Selecting On-Site Analytical Methods for Explosives in Water, Federal Facilities Forum Issue, EPA/600/S-99/002 , 1999, U.S. Environmental Protection Agency.

24. Jenkins, T.F., P.W. Schumacher, J.G. Mason and P.G. Thorne (1996) "On-site analysis for high concentrations of explosives in soil: Extraction kinetics and dilution procedures" Cold Regions Research and Engineering Laboratory (CRREL) Special Report 96-10.

25. U.S. EPA, Guidance for methods development and methods validation for the RCRA Program, June 1995.

26. Zeck, A., M.G. Weller, and R. Neissner "Characterization of a monoclonal TNT-Antibody by measurement for the cross-reactivities of nitroaromatic compounds", *Fresenius J Anal. Chem.*, 1999, **364(1-2)**, 113-120.

27. Crockett, A.B., T.F Jenkins, H.D. Craig, and W. E. Sisk (1998) "Overview of on-site analytical methods for explosives in soil" Cold Regions Research and Engineering Laboratory (CRREL) Special Report 98-4.

This page left blank intentionally.

APPENDIX A

POINTS OF CONTACT

Project Managers and Principal Investigators
Anne Kusterbeck
Code 6910
Naval Research Laboratory
Washington DC 20375-5348
Phone: (202)404-6042
Fax: (202)404-8897
E-mail: awk@cbmse.nrl.navy.mil

Lisa Shriver-Lake
Code 6910
Naval Research Laboratory
Washington DC 20375-5348
Phone: (202)404-6045
Fax: (202)404-8897
E-mail: lcs@cbmse.nrl.navy.mil

Reference laboratory data

All data may be found at Code 6900, Naval Research Laboratory, Washington, DC 20375.

ESTCP Program Office

901 North Stuart Street
Suite 303
Arlington, Virginia 22203

(703) 696-2117 (Phone)
(703) 696-2114 (Fax)

e-mail: estcp@estcp.org
www.estcp.org